

nature

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Sixty years of Soviet science

NEXT WEEK, on 7 November, the Soviet Union celebrates the sixtieth anniversary of the October Revolution. The discrepancy in dates, reflecting the change-over from Julian to Gregorian reckoning, neatly symbolises one of the major results of the Revolution—a commitment to implementing the results of modern scientific progress in the building of socialism.

For forty years, this policy was viewed from abroad as no more than an attempt to catch up with the burgeoning Western industrial economies. Soviet leaders' remarks about 'outstripping' the West were dismissed as mere propaganda—until the bleeps of *Sputnik 1* sent Western scientists running to their Russian primers and added to the information explosion an ever-increasing flood of cover-to-cover translations of Soviet journals.

One well-worn theme of Soviet writers on *naukovedenie* (the science of science) is that Soviet science must inevitably overtake the bourgeois variety, owing to its superior ideological basis. While we cannot support the idea that science, properly speaking, can belong to this or that political system, there does seem to be a distinct sociological phenomenon which may be termed 'Soviet science'—science carried out within and in the service of the Soviet State planning structure. Within this system science, like every other branch of production, is organised into neat five-year planning blocks. Research is envisaged officially as a search for 'solutions' to particular problems of the national economy, with the All-Union Academy of Sciences as 'coordinator for the whole scientific work of the country', and increasingly centralised control and 'unification' of academic institutions.

Unfortunately, it is precisely this centralised control which has led to major set backs for science in the Soviet Union, when high-level intervention by planners or ideologues overrode the scientists' desired research programmes. Lysenkoism, Stalin's attempt to ban quantum mechanics, the slow start in cybernetics (it appeared contrary to certain dicta of Marx and Lenin) are hardly a convincing argument for leaving decision-making in the hands of the State Planning Commission. Nor has this trend ceased. In recent years, the psycho-

logical theories of Snezhnevskii, who would attribute all mental disturbance and social nonconformity to schizophrenia, even when no schizophrenic symptoms are discernible, have begun to acquire the status of official dogma, and seem well on their way to repeating, in psychology, the disastrous course into which Lysenkoism led Soviet biology.

While the days are long past when a student might be asked to comment on the 'class significance' of a differential equation, ideological soundness remains a matter of utmost concern to the Soviet authorities. According to the latest (1976) regulations, a postulant for a higher degree must "combine a profound professional knowledge with a mastery of Marxist-Leninist theory and with the convictions of an active builder of Communist society". Official biographies and obituaries describe a scientist's minor Party offices as meticulously as his academic laurels. Organisers of international conferences know all too well that a Russian scientist of world repute will frequently fail to appear, being replaced by a junior colleague of mediocre scientific ability but greater Party commitment.

No one can deny the considerable achievement in Soviet science and technology over the last six decades, nor the fact that certain achievements, notably major irrigation projects, the space programme or the development of the resources of Siberia, would hardly have been possible without a centralised planning system. In the eyes of the comrade-in-the-street, science and scientists have acquired considerable charisma. Orders and decorations are regularly bestowed on deserving scientists by a grateful government.

Yet, by a typically Russian paradox, the constitutional position of scientists was until now anomalous in a State based officially on the union of workers and peasants. Only in the past month, with the publication of the revised constitution, has this been extended to read "workers, peasants, and intelligentsia". Recognised at last as part of the basis of the State, it is to be hoped that Soviet scientists will receive as an anniversary present a greater measure of freedom of movement and immunity from bureaucratic interference without which no science can effectively flourish. □



The lipid hypothesis: orthodoxy by default?

John Rivers, a London-based nutritionist, looks at the debate over diet and coronary heart disease

DR George Mann is a university teacher, a physician, a nutritionist and a heretic. He is one of the small number of outspoken opponents of the theory that the key to the modern epidemic of coronary heart disease (CHD) is the consumption of too much fat which is too high in cholesterol and has too low a ratio of polyunsaturated to saturated fatty acids. That this opposition should justify the term 'heretic' is not a reflection of the unchallengeable nature of the evidence supporting the 'lipid hypothesis', but the extent to which official gatherings of experts in different countries have made it national policy.

CHD is a sudden event, but as it only occurs in arteries narrowed and made less resilient by atherosclerosis, its origin must be sought in the factors which lead to years of deposition of cholesterol-rich atheromatous plaques. Virchow's observation over a century ago that the cholesterol in these plaques is derived from serum cholesterol suggested high serum cholesterol was the primary cause. Ample epidemiological evidence backs this view. The disagreement starts when one seeks the cause of high serum cholesterol.

As over 90% of our body cholesterol is endogenously synthesised, Mann dismisses dietary cholesterol as an important determinant of serum cholesterol. Rather, he suggests, one should look for factors which alter rates of synthesis or excretion. In his view the latter is crucial, and he notes a reduction in cholesterol breakdown is known to result from exposure to high levels of atmospheric carbon monoxide—hence the importance attached to smoking and pollution—and from consumption of *trans* unsaturated fatty acids.

Powerful evidence can be adduced to support his view that present dietary hypotheses of hypercholesterolaemia are mistaken. For example, a recent study of 2,000 subjects in Michigan found no significant differences in intakes of energy, total fat, unsaturated fat, saturated fat, or cholesterol between the upper and lower tertiles for serum cholesterol. What ultimately matters, however, is not the effect of diet on serum cholesterol but its impact upon mortality from CHD. Reviewing the dietary modification trials that have been conducted to date, Mann concludes that differences in mortality in primary or secondary clinical trials have yet to be shown. Although mortality from CHD is reduced in some, a diagnosis of the cause of death is relatively soft data, and the total death rate has remained constant. Mann argues that if CHD mortality really fell in these trials, mortality from something else must have risen for the total deaths to have been constant. He points out that this often seems to be cancer.

The idea that polyunsaturated fats predispose towards cancer dates from the studies of Pearce and Dayton (*Lancet* i, 464–467, 1974). Many nutritionists regard the idea as unproven—at the least the evidence about it is conflicting. But it is Morton's Fork. If, like Heyden (in *The Role of Fats in Human Nutrition*, Academic Press, 1975), one dismisses the apparent rise in cancer deaths as due to poor design of the trials, then the trials are also too poor to provide evidence about deaths from CHD. The trouble, says Mann, is that the experiments are not scientific. Scientists should design experiments to disprove hypotheses—dietary trials are still being done to confirm them. Because the lipid hypothesis is still not proven after 27 years, he says, the time has come for a rethink.

More troubling than Mann's scientific objections to the

lipid hypothesis are the effects that widespread acceptance of it has had. It has become self-sustaining: heretical opponents find it difficult to obtain research funds, and the medical hypothesis is being increasingly used as an advertising ploy—a massive vegetable oil industry needs evidence for the benefits of polyunsaturated fats.

Even more important, though, we may be doing exactly the wrong thing. Pursuit of the lipid hypothesis does not mean just swapping polyunsaturates for saturates. Polyunsaturates are too unstable, so much of the extra polyunsaturated fatty acids are provided by the substitution of soft margarines and stabilised vegetable oils for butter and animal fats. The trouble is, both changes lead to a dramatic increase in consumption of *trans* fatty acids, compounds whose known hypercholesterolaemic effect (Vergossen and Gottenbos, *The Role of Fats in Human Nutrition*) outweighs the putative benefits of polyunsaturated fats.

Butter contains 8% of its fatty acids as *trans* isomers, the product of biohydrogenation in the rumen. Vegetable oils, which like soft margarines are usually partially hydrogenated, contain *trans* fatty acid levels of up to 40%. Manufacturers of polyunsaturated oils and margarines may proudly state that they are high in polyunsaturates, but they do not state the levels of *trans* fatty acids, nor do they feel obliged to point out the crucial modification that those fatty acids may have undergone.

Like many of the critics of the lipid hypothesis, Mann has sought supporting evidence for his view from studies on the Masai. These meat- and milk-eating pastoralists have low serum cholesterol in spite of a high consumption of animal products. Mann argues that this can be expected—they have low levels of environmental pollution and eat little *trans* fatty acid. Nevertheless, his studies on 50 aortae obtained post mortem show that they do get atheroma but its effects are minimised by the tendency of the coronary artery to widen with age. This Mann attributes to heavy exercise, pointing to epidemiological studies which show a protective effect of heavy exercise. The question obtrudes itself. Is a dietary solution being sought because it is more acceptable than exercise?

It was sad that, to hear Mann talk, I had to attend a press gathering organised by the Butter Information Council, a public relations body representing the dairy industries of Europe. One must regret that he was not asked to present his views to a scientific audience, although they do have available a good summary in his latest paper (*New England Journal of Medicine* 297, 644–650, 1977). Our dietetic fate is in danger of being decided by a public relations battle between margarine salesmen and cowherds.

In the face of this, however, we cannot seek reassurance from the deliberations of the expert committees on the topic. Two successive UK committees on heart disease, under the aegis of the Department of Health and Social Security (*Diet and Coronary Heart Disease*, HMSO, 1974), and the Royal College of Physicians (*J. Roy. Coll. Physicians*, 10, 213, 1976), have come to diametrically opposed conclusions about the value of the lipid hypothesis. The evidence available to both was substantially the same. The latest report, from a US Senate Committee under Senator George McGovern, has recommended among other things a reduction by 30% in dietary total fat intake accompanied by a 50% rise in the consumption of polyunsaturated fatty acids. Such sweeping changes are only likely to be achieved by a substantial shift towards the use of vegetable oils and, according to Mann, may cause more problems than they solve. Not all that he says is convincing, but it is a powerful criticism of a view that is in danger of becoming orthodoxy by default. □

Improving bench work

Recent legislation affecting employment in Sweden has implications for research, as **Wendy Barnaby** explains

SHOULD research be regulated by the laws that govern industry, or is research a special kind of activity needing a different set of laws? The question arises in Sweden as a result of legislation over the past couple of years to strengthen the employee's position in relation to the employer, and it has a good number of scientists worried. For although a few very small categories of employees are exempted, the laws apply to all sorts of working places, and these include universities, research institutes as well as factories, bureaucracies and businesses. Given the different needs of these organisations, the question arises whether it is possible to design one set of rules which will secure the employees' place within them and at the same time improve the quality of their output. It is becoming obvious that the answer is no.

The Law on Security of Employment, which came into effect on 1 July 1974, is the one with the most dramatic effects on research. Its basic tenets are that employment is to last until further notice, which cannot be given without reasonable cause. An employee who is dismissed can contest the dismissal in a court of law and is generally entitled to keep his job while the case is being decided. The law thus abolishes the traditional principle that the employer can fire employees as he sees fit. It also contains various rules about the order in which employees are to be laid off in times when there is no work for them to do (a 'last in first out' basis) and specifies that an employee laid off for this reason has first option on new jobs with his old employer for a year after his dismissal.

The Social Democrats' intention in passing this law was to protect lower-paid workers whose jobs were at the mercy of market trends. It is a long way from the conveyor belt at Volvo to university laboratories, yet the law applies to people working at both. In practice, it has meant that university lecturers have gained security in their jobs instead of having to re-apply for them annually or every third year. Only one category is still competitive, that of research assistant (*forskarassistent*), a position filled by a newly-graded PhD who has to re-apply for it after three years. The reasoning behind this exemption, according to a representative of the academics' union at one of Sweden's larger universities, is that possibilities should be kept open for

PhD's who want to go on researching and teaching.

On paper some flexibility has also been maintained with the prestigious position of docent, the last of the stepping stones to professorial rank. A docent's contract lasts for three years, after which time it may or may not be renewed. Because most docents are in their forties and fifties and have probably spent all their working lives at the university, it looked too callous to throw them out on to the street if they had become stale in their research. Under the law, therefore, the university is obliged to create for any docent whose contract is not renewed a new position at equivalent salary but not involving research. The funds for this are to come out of the university's research revenues. It is an extremely expensive way out, so expensive that it is hardly ever resorted to. In practice, docents have tenure.

Thus, after the statutory six months probation period, all members of a university department except research assistants have tenure, with docents' security practically assured. The results are not hard to imagine. No incompetents can be fired, so no new blood (except research assistants) can be hired. There is minimal circulation of staff and, many complain, a loss of creative atmosphere. The Rektor of Uppsala University, Professor Torgny Segerstedt, says there is an enormous risk that research will become very static.

Its effects will be felt in other ways, too. "I don't dare hire a new secretary", says one professor, "because I know I'll never be able to get rid of her if she turns out to be no good." A microbiologist complains: "I have a good research project I'd like to start, and I've even got a grant for it. But I can't hire a laboratory assistant. We could be stuck with him for ever". In theory, the law allows short-term contracts for specialists hired for a particular task, but in practice this may be impossible to enforce because it is difficult to identify a scientific project as a special job being done in an institute concerned with the same area of research. An assistant employed on, say, a two-year project who wants to stay at its completion would probably succeed in proving that he has taken part both in the specialised project and in the general work of the institute, and would then be considered to have been employed by the institute as



All affected:
lab assistant to glass-blower

such.

Against defenders of the law who say that research council grants will keep some turnover in projects and personnel, it is argued that such grants will become just as tied up as ordinary projects because they are formally given to the universities which then pass them on to the successful applicants. When any project is over, therefore, the researchers could claim that it had been the university, not the Council, that had been employing them, and demand to stay. At this point the university would be obliged to see if it could find the displaced researcher a job he was competent to do, even if he was not enthusiastic about it.

Thus, a research biologist could be offered a job tutoring biology to first-year students, if such a vacancy were available. If no department had any suitable vacancy, the university would be entitled to fire him on the grounds of there being no work for him to do. In practice the university, acting totally within the law, has retained displaced researchers by firing the most-recently employed members of staff in jobs suited to the displaced researchers' qualifications. In small institutes, however, the regular staff normally dependent on research grants have to leave if their grants are not renewed.

It is difficult to see how, under the law, any research project could be abandoned while the researchers wanted to carry on with it. This raises questions about the whole direction of research: how to drop enquiries which are no longer interesting or profitable, and how to swing research into new channels. Problems would arise not so much at the end as at the beginning of new projects. Unless job security for skilled technicians can be provided, they won't be there in the first place.

Working life law

The other law making itself felt at research institutes is the Act on the Joint Regulation of Working Life (*Medbestämmandelag*, MBL), which came into force at the beginning of this year. Its purpose is to increase union participation in decision-making. It does this partly through revising the rules about decision-making, so that the management has to inform and negotiate with the union if any major changes are contemplated, and partly through widening the scope of issues about which the union is entitled to have a say. The most important provision in this respect is that unions and management who have a collective agreement on wages and conditions of work—which nearly all have—should negotiate agreements on joint regulation, which will set out the rights of the workers to organise and assign

work, hire and fire employees, and in general carry on the work of the organisation. The management is also required to provide the unions with information about financial and production matters and personnel policy.

Because most research establishments are financed by the government, the effects of MBL on research are somewhat modified by the Public Employment Act (*Lag om Offentlig Anställning*), which is designed to give public employees the benefits of MBL without trespassing on that part of their work which, because of democratic norms, the public at large has the right to preside over. As far as research institutes go, this means that the employees would not, for example, be able to change the general focus of research as laid down by parliament in the institute's statutes. It also means that, although the management must inform and negotiate with the union, it is not obliged to accept union opinions in questions considered to be in the public domain. In the absence of agreements on joint regulation, MBL in research institutes is at the moment largely a matter of an increase of information and negotiation. But the new system does take up a lot of time.

In some institutes, the atmosphere has been poisoned by union-management disagreements over how MBL should work. "Academic unions are young and inexperienced in comparison with industrial unions", says one physics professor who has had a hard time of it. "They minimise the role of informal contacts and consultations which are so important in industrial negotiations". Although running-in problems are giving some of

the unions a bumpy ride, all agree that their power has increased. "Research is expensive", comments one employee, "and if everybody has to take part in all decisions and planning it will cost more money, time and paper. With the help of MBL, employees might insist on comfort in laboratories instead of equipment. On the other hand, MBL based on public opinion might enforce the financing of research which might otherwise not be carried through".

Different disciplines envisage different problems. "Take an experimental team", says another physics professor. "It is made up of, say, a professor, an assistant professor, a PhD, a couple of graduate students and some technicians. It is a small group and its members usually get together and decide on working practices that suit them all, which helps to mobilise creativity and enthusiasm. But the members of these groups are represented by different unions, who are not involved in the immediate situation but who will now decide on what is to happen there. Direct democracy is being substituted by indirect, and it won't help the atmosphere in the laboratories".

Both laws are quite new, and their full impact on research may not be clear for a few years yet. What does seem obvious is that, especially in the case of the Security of Employment Act, it does research little good to be swept up with industry and put into the same legal basket. Some see Swedish research being irreparably damaged. The government is aware of the difficulties and is to conduct a review. In the meantime, Sweden will remain a focus of attention for external as well as internal interests. □

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USA

Security conscious

The Worldwatch Institute, the private Washington-based research organisation concerned with global problems, published its latest pamphlet last week. Chris Sherwell reports

"THE present deficiency of assured energy resources is the single surest threat that the future poses to our security and to that of our allies", the US Secretary of Defense, Harold Brown, told a gathering of business leaders in the United States last week. For a namesake of his at the Worldwatch Institute, the timing was near-perfect. Lester Brown, Worldwatch's president, was about to release *Redefining National Security*, which considers why energy and other global problems pose a new threat to national security that only a new approach can combat.

These new threats to security, Brown says, "arise less from the relationship of nation to nation and more from the relationship of man to nature". From his discussion of them he concludes that the traditional military concept of national security is no longer adequate for an interdependent world facing global ecological and economic problems. Political leaders have to realise that national security is meaningless without global security; countries must address the problem, he says without amplification, "cooperatively".

OTA appointment

THE position of Director of the Congressional Office of Technology Assessment (OTA) has been given to Dr Russell W. Peterson. He is president of a lobbying organisation concerned with global issues called New Directions and formerly chairman of the Council of Environmental Quality and Governor of the State of Delaware.

The choice follows several months of speculation and political infighting sparked off by allegations that Senator Edward Kennedy was trying to take control of the OTA and had engineered the resignation of its founder and first Director, Emilio Daddario. These allegations were strongly contested at the time and will be further diminished in credibility by the fact that Peterson is a Republican with a reputation for independence. In addition, Kennedy has been scrupulous in avoiding any appearance of interference in the selection process.

Other nominees for the job were Russell Train, former head of the Environmental Protection Agency, Daniel Desimone, presently the deputy director of OTA, and John Sawhill, former head of the Federal Energy Administration.

Sandy Grimwade

The discussion itself ties together several well-known threads of argument that have already appeared in other Worldwatch publications. Thus, in respect of energy, Brown says that an oil-dependent world, in a nuclear limbo and facing climate alteration with heavy use of coal, must act in concert to produce a timetable for a transition to renewable resources. The world's biological systems, on which the global economy depends, says Brown, are similarly threatened by excessive human claims: fishery catches exceed the long-term sustainable yield, tree-cutting exceeds the regenerative capacity of forests, grasslands deteriorate as livestock and human populations increase, and erosion damages croplands as population pressures mount.

Brown takes his arguments further. Humans can inadvertently or intentionally alter global climatic patterns, he states, causing agricultural output to shrink and adversely affecting the survival prospects of hundreds of millions of people. And a "basic transformation" in the world food economy in the 1970s means that the global balance between demand and supply remains delicate. This, says Brown, is attributable to agricultural shortcomings in Eastern Europe and the USSR and agricultural mismanagement elsewhere, rapid population growth and "negative ecological trends"—deforestation, overgrazing, desert encroachment, soil erosion and flooding.

Finally he argues that economic threats to security—simultaneous inflation and unemployment on a global scale—aggravate social divisions, not least through a worsening income distribution. Taken with the other factors, which also translate into economic stresses, the military's role in securing a nation's well-being and survival is "relatively less important than it once was". But political leaders "perceive the new threats dimly".

Outside the optimists who through faith in man's ingenuity see no problems ahead, doubts about all these arguments will come from those who believe ecological and environmental problems will resolve themselves at great human cost regardless of political intervention, and from those who believe in political intervention but have little hope for it. Doubts of the first kind may simply be unacceptable to Brown, but the second kind pose a difficulty. After all, is it really the case that if *all* nations don't hang together they will hang separately? □

BRITAIN

Help at the margin

UK Research Councils have received a small windfall. Chris Sherwell reports

WHEN the UK Advisory Board for the Research Councils (ABRC) holds its regular meeting at the end of next week, it will consider how to advise Shirley Williams, the Secretary of State for Education and Science, on the allocation of an extra £4 million among the country's research councils. The sum was added to the department's 1978-79 Science Budget by the Chancellor of the Exchequer, Mr Healey, in his 'mini-budget' last week. Science administrators who recognise, in the words of one, that "it is the margins which cause us the misery", greeted the news enthusiastically.

It is not yet known whether the extra cash represents a once-and-for-all increase or the beginning of a longer term upturn. No indication of what precisely will be happening in the period after 1978-79 is yet available. This means that research councils must prepare options: greater expenditure on capital projects or special programmes will make different demands from improved research grants, for example, which would be recurrent. Last year's forward look means that a fair amount was already known of what councils might want to do with the extra cash.

Mrs Williams has asked the ABRC for its "urgent" advice. If a decision is not made next week, the next meeting is set for 9 December. □

NETHERLANDS

Limited progress

The position regarding recombinant DNA research in the Netherlands is still not clear. Casper Schuurings reports

THE old Dutch government, still in office five months after the 25 May elections, has decided to start preparing legal rules for recombinant DNA research in the Netherlands. The minister of Health and Environmental Protection, in a letter to parliament, says that so long as not enough is known of the positive and negative consequences of this research, no concrete judgment can be given about the contents of such legislation. "There is also not enough known about the social relevance of this research and therefore of the priority it must have in research generally". In the meantime,

he added, the greatest possible reserve should be exercised.

Practising scientists have expressed their disappointment and concern over this lack of clarity about the policy the government foresees. They fear that the degree to which the Netherlands is falling behind in this field will become greater, and there is concern about the matter of contracts between researchers, their universities or institutes and the Commission on Genetic Engineering of the Royal Netherlands Academy of Sciences. The Commission published a report on 30 March which concluded that recombinant DNA research should be developed with stringent precautions and according to clear regulations contained in legislation which should be implemented quickly.

The government is not following this advice exactly, but the signing of research contracts has not ceased. Researchers at the Free University of Amsterdam signed a contract with their own board and with the commission, and the group is about to start work. Safety control remains with the university itself. Groningen University has also signed a contract, but is further from starting.

The commission's report proposed two commissions, one concerned with safety measures, the other with ethical aspects. The minister, however, announced a new and broader *ad hoc* commission to take over a part of the task of the Academy's commission, which is now composed only of experts. This *ad hoc* commission has to register, advise and indicate the potential dangers of the research in different categories of risk.

The League of Scientific Researchers last month reacted critically to the report, saying it "underestimated and played down" the risks of recombinant DNA research. "The safety measures proposed by the commission are suggested more by their feasibility than by their demonstrated safety", the league said, and praised the minister, who will not return in the new cabinet, for perceiving the need for a broad discussion and for a broader commission. The league recommends a "freezing" of the experiments, and urges the government to stimulate discussion involving scientists, politicians, the lay public and others.

No requests have come so far from industry to start recombinant DNA research. Unilever announced earlier that it was making preparations, and Gist-Brocades is known to be doing the same. But union members are unhappy about this. One of the largest unions in the country has written to three ministers requesting a stop to the research while no legislation exists.

COMECON

● Protests against the US decision to produce neutron bombs have occupied an important place in the Comecon media over the past three months, with special emphasis being placed on protests by scientists or scientific bodies. Not surprisingly, such protests have so far come from the scientific establishment, but more recently even 'dissident' opinion was called in as support, with an interview on Prague radio with Academician Andrei Sakharov in Moscow.

Sakharov's opposition to nuclear warfare is well known. Early in his career of protest he turned over his savings from his work on the Soviet hydrogen bomb to cancer research. It was his apprehension of the danger of nuclear catastrophe which led to his first major *samizdat* essay, *Progress, Coexistence and Intellectual Freedom*, in 1968. As a Nobel Peace prize-winner, Sakharov would surely have been approached even earlier in the current campaign were it not for his reputation of dissidence.

The interviewer attempted to dissociate 'Sakharov the Soviet scientist' from 'Sakharov, the number two Soviet dissident', alleging that in this second *persona* Sakharov merely rubber-stamped the views of his wife—the implication being that while she was in Italy for medical treatment, people could witness the 'real' Sakharov. But he refused to comment on the neutron bomb, save in the context of nuclear armament as a whole. The question of this one particular weapon, he said, must not be misused for propaganda purposes and must not be given moral and emotional overtones. He also suggested that it was fear of a (conventional) Soviet attack on Western Europe which had led the USA to work on the neutron bomb, and that, although convinced that neither the Soviet Union nor any nation wants a war, twice in this century wars had broken out "irrationally".

This latter statement, which contradicts the classical Marxist interpretation of twentieth century history, was characterised by the interviewer as "ludicrous". Sakharov himself was said to bear the "evil marks" of dissident thinking. But in spite of this and Sakharov's refusal to take action against one specific weapon, the interview was broadcast and presented as something of a journalistic scoop for the current anti-neutron bomb campaign.

● *Interkosmos* 17, launched on 24

September, inaugurated a new generation of Comecon space probes. Previous *Interkosmos* craft had been small, relatively light satellites used for limited studies of cosmic and solar radiation, the magnetosphere and ionosphere. The satellites were not properly stabilised with respect to the Sun, and hence the data obtained was only of a fairly general type.

Accordingly, a new type of satellite, called an "automatic general-purpose orbital station" (AGPOS) was developed for the *Interkosmos* programme. A prototype was launched last year as *Interkosmos* 15; *Interkosmos* 17 is the first operational AGPOS. Designed for long-term automatic operation, the AGPOS includes a special orientation system and can even be equipped with an orientation platform to carry instruments for the high-accuracy investigation of solar flares.

Although the *Interkosmos* programme theoretically includes all countries of Comecon, six out of the twelve on-board experiments were provided by Czechoslovakia, including a laser reflector to determine the distance between the satellite and earth, a silicon cosmic-ray detector, and devices to measure the mass and velocity of meteorites. Czechoslovakia is playing an increasingly large role in the *Interkosmos* programme. Czech trainee-cosmonauts preparing for the manned *Interkosmos* programme are reported to have completed their theoretical work and are training in simulated conditions. One of them is expected to go on a mission with a Soviet cosmonaut in 1978.

In addition to participating in *Interkosmos*, Czechoslovakia has also provided experiments for the Soviet *Prognoz* 4, 5 and 6 satellites which have highly elliptical orbits. The launching of an independent Czechoslovak satellite, and the inclusion of Czech instruments on Soviet lunar and interplanetary probes is also envisaged.

At the same time Bulgaria, hitherto a relatively minor participant in *Interkosmos*, is also planning an independent space programme to be associated with the 1,800th anniversary of Bulgarian statehood next year. A recent decree of the Council of Ministers approved a regulation introducing "a nationwide programme to study and utilise outer space, and a preparatory programme to launch into outer space the project 'Bulgaria 1300'".

Vera Rich

IN BRIEF

Food action urged

A publication from Earth Resources Research, the research arm of Friends of the Earth in Britain, urges government action using taxes and subsidies to encourage consumption and production of foods that strengthen consumers' health, minimise environmental pressures and make the best use of food resources.

Changing Food Habits in the UK, by Chris Wardle, argues that the two main government regulatory bodies, the Food Standards Committee and the Food Additives and Contaminants Committee, should be provided with their own scientific staff and research facilities. There should also be, he says, an intensive programme of consumer education on nutrition, the introduc-

tion of nutrition labelling, and an independent body to oversee all forms of food advertising.

German technology assessment?

The opposition CDU/CSU parties in the West German Bundestag have proposed a motion to set up an office of technology assessment. They proposed this once before, in 1972. The new motion follows years of discussion and calls for the office to be set up within the existing Bundestag administration and for expert knowledge to be gained by placing orders both at home and abroad. It is regarded as important that Federal government offices should call on foreign experts for advice. The Bundestag will deal

with the motion over the next few months.

Europe compact suggested

Professor Peter Odell, appointed earlier this month as a part-time consultant to the UK Department of Energy, said this week that the restraint on oil and gas development imposed by the lack of a West European agreement should be overcome by a compact between the exporting and importing countries concerned.

In a joint paper with Dr K. Rosing, his colleague at Erasmus University, at an Institute of Fuel conference, he said that Europe's strategy for oil and gas production is "constrained more by politics and institutions than it is by the likely size of the resource base".

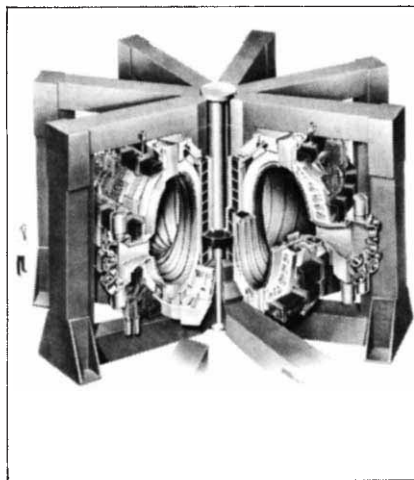
THE EEC's Joint European Torus (JET), which was finally given to the UK's Culham Laboratory last week, is essentially a device to confine the ionised particles of a plasma long enough for fusion reactions to occur between them. The two light elements most suitable for use in fusion machines are deuterium and tritium. The former is plentiful—it can be extracted from seawater—but the latter does not occur naturally and is made by bombarding lithium, a naturally occurring element, with neutrons.

The most successful machine designed so far for confining plasma, the tokamak, was developed in the USSR and forms the basis of the JET design. The plasma is confined within an evacuated D-shaped torus by a magnetic field, created by field coils linking the torus, and by a current flowing through the plasma. For the energy obtained to be greater than that put in, plasma temperatures have to be about 100 million °C and the product of plasma density and confinement time has to be greater than 10^{14} cm⁻³s.

It is the attainment of such stringent operating conditions that has made fusion so difficult to harness. JET aims to achieve higher temperatures and plasma densities and longer confinement times than any of its predecessors. According to the original plan it was to take five or six years to build. Experiments were then to proceed for two to three years, by which time they would have produced good enough results for a prototype reactor to be designed.

There is doubt, however, about the realism of this timescale. In spite of progress so far, it is unlikely that

the road to what has been called a cheap, inexhaustible and clean source of energy will be smooth. As well as technical problems, those of fuel

Fusion's promise**BACKGROUND**

supply, waste disposal and potential radiation leaks will have to be tackled, even though these may not be so great as those for fission reactors.

The radiation hazard stems from the possible release of tritium, a radioactive gas which will have to be contained within the reactor. Tritium for the fusion process will be manufactured as hot neutrons interact with lithium in the container walls. Constant bombardment of the walls will make them radioactive and wear them out so that they will have to be replaced from time to time. Even

though the products of the fusion process will not be radioactive and will pose no disposal problem, the old reactor walls will. The fuel supply problem will arise when world reserves of lithium run low. Present estimates put the reserve at about the same as uranium.

The enthusiasm for fusion as the answer to the world energy problem is based on the knowledge of what it could do, theoretically and not on what it actually might do on a large scale. If the fusion reaction were between deuterium and deuterium, for example, the problem of lithium supply would vanish. If it were between deuterium and helium-3 an added advantage would be that neutrons would not be produced and the problem of damaged radioactive walls would vanish. Similar advantages would be gained by using hydrogen and boron. But all these alternative fuels have their drawbacks when it comes to operating a large scale fusion machine, mainly because they would need even higher temperatures to ignite fusion reactions. Deuterium and tritium are at present the only promising fuels.

The outlook for JET itself, however, is fairly bright considering the two years delay. While politicians were negotiating, the team at Culham was building one-off pieces of equipment and refining the design. It has already placed phased contracts for some of the larger parts including the coils for the toroidal field. The next step is to appoint a head of project and management committee. Firm contracts for the rest of the parts will be placed throughout Europe and construction should begin within a few months.

correspondence

Petition for Argentinian scientists

SIR,—Some colleagues in our cancer centre are very concerned by the actual situation in Argentina and we have decided to propose the adjoining text for the approval of the international cancer community.

The undersigned cancerologists are very concerned by the situation in Argentina where personal security is increasingly threatened, fundamental rights are denied and where repression and arbitrary police action severely effect the scientific community ('Repression in Argentina: Scientists caught up in tide of terrors' *Science* 194, 1397; 1976; and 'More on Argentina' *Nature* 263, 452; 1976).

These cancerologists refuse to participate under such conditions in the twelfth International Cancer Congress, which is scheduled for 5–12 October 1978 in Buenos-Aires, and ask all the members of the international scientific and medical community to join them in their refusal.

The undersigned also refuse to participate in meetings organised in any country subject to police repression and where the rights of man are systematically violated.

We invite physicians and scientists who share these opinions to join us in this protest by signing this petition. Copies of the petition will be made available on request to Dr J-C. Salomon, Institut de Recherches Scientifiques sur le Cancer, CNRS, B.P. 8, F 94800 Villejuif, France.

A similar action is underway among United States cancerologists.

LOUISE HAREL

JOSE URIEL

JEAN-CLAUDE SALOMON

Villejuif, France

Exchange agreement

SIR,—I was glad to see Vera Rich's article (13 October, page 553) about the new exchange arrangement between the Royal Society and the Academy of Sciences of the USSR, but sorry to note that it contained several misleading statements. Firstly, the seven senior and four junior visits she referred to are in the *current* agreement, and are replaced in the new agreement, to come into effect on 1 April 1978, by a more flexible arrangement for a total of 49, not 40, man-months. The Royal Society hopes that this number will be increased and that the more flexible exchange agreement will lead to wider support for visits in both directions.

Vera Rich's comparisons with UK–Poland exchanges are also highly misleading as the figures she quotes for UK–USSR visits refer only to those under the Royal Society–Academy exchange agreement and do not refer to many visits under other auspices, while those for UK–Poland are evidently all-inclusive; Royal Society–Polish Academy visits account for only about ten each way.

R. W. J. KEAY

The Royal Society,
London, UK

Which diet?

SIR,—Colin Blythe and Howard Rush in their article 'What kind of food policy?' (4 August, page 386) mention the confusing advice available "to individuals who wish to follow sensible eating regimes".

Over past years ever-increasing doubt has been cast upon the validity of the theory that animal fats and cholesterol in human food cause arteriosclerosis and its resultant disorders. Even drastically reducing fat consumption and substituting animal fats to a large extent with distasteful plant oils of high polyene acid content, does not restore blood cholesterol levels to normal, nor bring about a retrogression of arteriosclerosis.

The fat theory offers us no explanation of the fact that in primitive populations, whose diet contains a very high proportion of animal fats, the incidence of arteriosclerosis and abnormal blood fat levels is practically nil. The diet of such people (Massai, other nomadic tribes, Eskimos) usually contains very little carbohydrate. Might is not therefore be the carbohydrates and not the fats that are responsible for the cardiovascular disorders connected with other types of civilisation?

Support for this idea is provided by observations on the cholesterol levels in

subjects of various age groups on a low-carbohydrate diet (70 g daily). Data collected from over 300 individuals have revealed that the cholesterol can be brought down by 50 mg% in 39% of the patients over 60 years of age, in 52% of those between 40 and 60, and in 67% of the group under 40. In the under-forties, restriction of carbohydrate intake can bring about permanent normalisation of the values within a short period of time, if we consider the average values as shown in the figure.

The possibility exists that every case of hypercholesterinemia could be reversed, or even avoided in the first place, if a low-carbohydrate diet were to be adopted in time. If we were to consume as little carbohydrate as the above mentioned primitive peoples, hyperlipidaemias and arteriosclerosis would probably not occur. Such disorders develop over the years under the influence of unphysiological quantities of carbohydrate (and their reversibility declines with advancing years) until the point is reached at which animal fats and cholesterol in the food bring about a rise in blood cholesterol levels. The efficacy of a low-carbohydrate diet in reducing overweight and in correcting metabolic disorders has already been established. Perhaps it would now be worthwhile considering the substitution of the fat theory by a carbohydrate theory in explaining the diseases connected with human civilisation.

WOLFGANG LUTZ

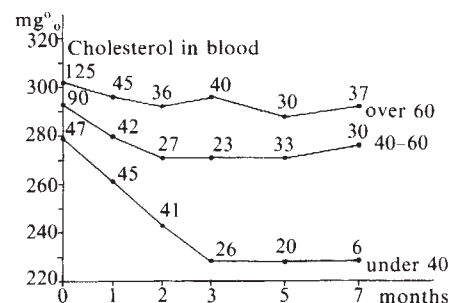
Facharzt für Innere Medizin,
Salzburg, Austria

On missing the point

SIR,—It is surprising that Bowne (13 October, page 556) finds some research ridiculous since it involves the discussion of polygenes, which are "unknown". It is surely a basic part of the scientific method to describe observed phenomena (familial resemblance for metrical characters) in terms of abstract entities (polygenes). Current work in genetics is not analogous to von Leeuwenhoeck studying ribosome structure, since the phenomena accessible to him did not suggest the idea of the ribosome.

G. A. T. MAHON

Trinity College,
Dublin, Ireland



news and views

Aether drift detected at last

from Michael Rowan-Robinson

A GROUP of experimenters from Berkeley (Smoot, Gorenstein & Muller, *Phys. Rev. Lett.* **39**, 898), using a U-2 aircraft in the unfamiliar mode of looking upwards, seem to have decisively detected the motion of the Earth with respect to the cosmic microwave background. This radiation, with the spectrum of a 2.7 K blackbody, is believed to be the relic of the 'fireball' (radiation-dominated, optically thick) phase of the Universe. The isotropy of the microwave background to one part in 10^3 has been the strongest argument in favour of the homogeneous, isotropic models of the Universe dreamed up by Einstein, de Sitter, Friedmann, Eddington and Lemaitre in the 1920s. But it has long been expected that a simple dipole anisotropy of order 10^{-4} to 10^{-3} should be found, due to the random motions that galaxies have with respect to each other and to the cosmological frame of reference (the frame in which the expansion of the Universe looks exactly isotropic, according to the models). The radiation should look slightly hotter in the direction we are travelling towards, and slightly colder in the direction we are travelling from, by an amount $\Delta T/T \approx v/c$, due to the Doppler shift. Failure to detect this effect would put us in the uncomfortable position of happening to be exactly at rest with respect to the cosmological frame. It was Michelson and Morley's failure a century ago to detect a contribution from the motion of the Earth to the local velocity of light that led to the downfall of aether theories and ultimately ushered in the relativistic age. A completely isotropic microwave background might have been equally revolutionary, but the Berkeley workers seem to have saved us from this fate. However the magnitude of the velocity deduced for the Milky Way, 600 km s^{-1} , is so large as to

throw existing ideas about our cosmic environment into disarray.

The Berkeley group did their experiment in the NASA-Ames Earth Survey U-2 aircraft and made eight flights over a period of 5 months. Two radiometers working at a frequency of 33 GHz ($\lambda=9 \text{ mm}$) point at positions 60° apart on the sky, 30° on either side of the zenith, and the difference between the signals in the two receivers is recorded. An isotropic background would therefore give zero output wherever you looked. The plane flies on a level path, banking and reversing its direction of flight every 20 min. The whole apparatus is rotated through 180° every 64 s in order to eliminate asymmetry between the two radiometers. Anisotropy in the Earth's atmospheric background is monitored with a second receiver working near the strong oxygen line at 5 mm. The effect of any rapid changes in the gain of the radiometers is reduced by rapid switching between them at 100 Hz.

About 5% of the sky was sampled, all in the Northern Hemisphere. The results agreed well with what would be expected due to motion of the Earth: a temperature excess in one direction, a deficit in the opposite direction, and in between a cosine dependence on the angle between the direction of the peak and the direction of observation. The derived velocity, after correction for the motion of the Earth round the Sun and other minor effects, is $390 \pm 60 \text{ km s}^{-1}$ towards a direction R.A. (Right Ascension) $= 11.0 \pm 0.5 \text{ h}$, declination (dec.) $= 6^\circ \pm 10^\circ$ (galactic longitude (l) $= 248^\circ$; latitude (b) $= 56^\circ$). This is within 2σ of the recent preliminary ground-based measurements of Corey & Wilkinson (*Bull. Amer. Astr. Soc.* **8**, 351; 1976), which are of lower accuracy. When corrected for the motion of the Solar System round the Galaxy, the net velocity of our Galaxy with respect to the microwave background is 603 km s^{-1} in direction R.A. $= 10.4 \text{ h}$, dec. $=$

-18° ($l=261^\circ$, $b=33^\circ$). Apart from this dipole anisotropy the microwave background is isotropic to one part in 3,000 and limits can be set on the rotation of the Universe ($<10^{-9}$ arc s per century) and on the average energy-density of long-wavelength gravitational radiation (less than the so-called "critical" density which would close the Universe).

What are we to make of this? The authors note that the velocity they have found conflicts with various attempts to measure our velocity with respect to nearby galaxies, but offer no explanation of this. With respect to the Local Group of galaxies, the motion of the Solar System hardly differs from that expected due to our circular motion round the Galaxy. This suggests that the whole Local Group has to be moving along together at this velocity of 600 km s^{-1} with respect to the microwave background. And this velocity is more than ten times the residual random motion of galaxies within 20 Mpc about the Hubble flow, so that most nearby galaxies, including the Virgo cluster of galaxies, would seem to have to move along together at this velocity. The Universe may be much more inhomogeneous than we have realised till now, and we may have to be careful about interpreting the expansion time-scale we measure locally as the age of the Universe.

The Berkeley measurement also conflicts with the velocity of the Earth with respect to more distant galaxies (at several hundred Mpc) determined by Rubin *et al.* (*Astr. J.* **81**, 687; 1976). They also found a velocity of about 600 km s^{-1} , but in a direction almost at right angles to the velocity with respect to the background. This 'Rubin-Ford effect' may not seem so surprising now. The matter in the Universe may be divided up into quite large 'metaclusters' or 'superclusters' in rapid motion relative to each other and to the cosmological frame.

It is unlikely that this new result will dent the hot big bang picture of the

Universe very much but our ideas about our cosmological locality are in for some revision. The Berkeley group have made an important experiment, which is clearly worth repeating. A

good test would be to do a similar series of flights in the southern hemisphere but perhaps U-2's are still not all that welcome outside US airspace. □

Visualising jumping genes

from T. Cavalier-Smith

DNA molecules are not an inert repository of genetic information, but are frequently chopped, spliced or modified by enzymes. This occurs not only during genetic recombination, but also during replication, repair and sometimes even during nuclear differentiation; for example, extensive chopping of DNA into pieces occurs during macronucleus formation in hypotrich ciliates (Lauth *et al.* *Cell* **7**, 67; 1976). Usually these changes leave the linear order of genes unaltered, but sometimes genes may 'jump' from one part of the genome to another. The first evidence for 'jumping genes' (more properly called transposable or translocatable genetic elements) came long ago from Barbara McClintock's studies of controlling elements in maize (*Proc. natn. Acad. Sci. U.S.A.* **36**, 344; 1950). The study of the molecular basis of transposability is now possible as a result of the discovery over the past few years of a variety of transposable elements in *Escherichia coli*. The simplest of these are the insertion sequences (IS), short segments of DNA of defined length and sequence which can become inserted into a larger DNA molecule by a recombination process distinct from *E. coli*'s normal recombination system. They were first discovered as a result of the strongly polar mutations they cause when inserted into the *lac* and *gal* operons, but have since been found in other chro-

mosomal locations and on drug resistance (R) plasmids (for reviews see Cohen *Nature* **263**, 731; 1976; Kleckner *Cell* **11**, 11; 1977).

Electron microscopy of the molecular hybrids formed after denaturation and reannealing of DNA containing insertion sequences showed that there were four distinct sequences: IS1 of about 800 base pairs, and IS2, 3 and 4, each about 1,400 base pairs long. Chow (*J. molec. Biol.* **112**, 611; 1977) has now used electron microscopy to throw some light on their distribution in the *E. coli* genome. Her method (much used in the past few years to study palindromes or inverted repeats in eukaryote DNA) was simply to allow the denatured DNA fragments (in length a few per cent of the total genome) to reanneal for such a short time that most of the duplexes formed were formed intramolecularly by a DNA strand folding back on itself. A single DNA strand can pair with itself in this way only if it contains at least two identical sequences that are inverted with respect to each other. If these inverted repeats are adjacent (forming a palindrome) renaturation produces a double-stranded hairpin; if they are separated by a spacer that cannot pair with itself, a double-stranded stem bearing an unpaired loop at one end is formed. Both hairpins and stem-loop structures are found in abundance in rapidly renatured eukaryote 'snap-back' DNA.

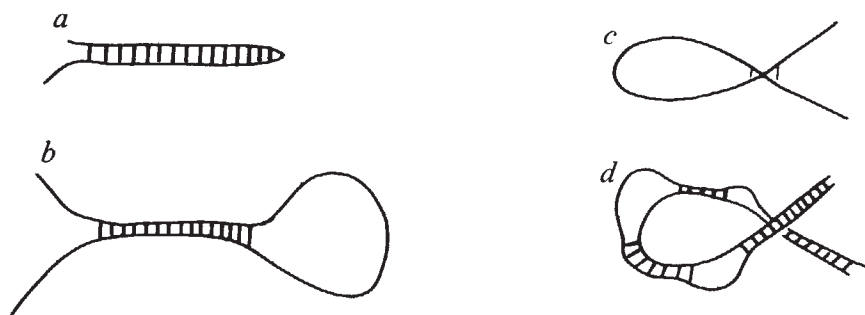
In *E. coli* DNA Chow found no hairpins (which confirms earlier work), but various stem-loop structures were

present, indicating that *E. coli* DNA does contain some inverted repeats long enough to be observed in the electron microscope (but only ones where the two sequences are separated by spacers). The duplex stems of the stem-loop structures fall into four discrete size classes, of which one corresponds to IS1 and one to IS2-4 (duplexes shorter than 400 base pairs were discounted as possible artefacts). The loop lengths are more variable but also fall into distinct classes, most being either 22,000 or 27,500 bases long, indicating a regular arrangement of the inverted sequences on the chromosome. By making various assumptions (including the identity of the 800-base-pair sequences with IS1) Chow estimates that there are a minimum of 14 IS1, and 10 IS2-4 (as well as 6 and 12 respectively of the 500-base pair and 1,000-base-pair sequences) in each genome. A similar study by Deonier and Hadley (*Nature* **264**, 191; 1976) revealed inverted repeats only in the 1,300-base-pair size range, suggesting that the IS sequence content of *E. coli*'s genome differs from strain to strain.

Some of the antibiotic-resistance genes carried on R plasmids have turned out to be bounded by insertion sequences (often inverted with respect to each other). The complete genetic unit is termed a transposon and can translocate from one plasmid to another and to the bacterial chromosome without the need for a complementary sequence to be present on the host DNA molecule. It is thought that interaction between the two inverted sequences plays an important part in this translocation.

In view of the apparent importance of inverted repeats one wants to be able to identify them easily. If they are longer than about 200 base pairs electron microscopy of the snap-back DNA fraction shows hairpins or loop-stem structures, but shorter inverted repeats do not show up in this way. Much shorter inverted repeats do exist, however, for example bacteriophage Mu DNA contains a segment of DNA (the G segment—3,000 nucleotide pairs long) which is flanked by inverted repeats each of less than 50 nucleotide pairs. In the prophage (but curiously not in the infective state) the G sequence readily inverts, presumably as a result of recombination between the inverted repeats. These short inverted repeats are seen by electron microscopy after denaturation and self-annealing as a 'cross over' of the two strands at a reproducible position (as in the letter α). But in DNA less well characterised than Mu it would be hard to distinguish between such cross overs caused by limited base pairing

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The four structures visible in the electron microscope after reannealing denatured DNA containing inverted repeats. a, Hairpin; b, stem-loop structure; c, α -structure with defined crossover; d, underwound loop formed by partial pairing of two complementary α -structures.

and the chance lying of one strand on top of another normally seen in the electron microscope.

Detection of such short inverted repeats will be made much easier by the recognition by Broker *et al.* (*J. molec. Biol.* **112**, 579; 1977) that following denaturation and reannealing they give rise to a characteristic structure which they call an 'underwound loop.' When a single molecule containing two short inverted repeats anneals with itself it forms a structure with a single-stranded loop and two single-stranded tails (like an α). If the DNA is left to reanneal long enough the loops of separate molecules will come together in complementary pairs. But this pairing must be incomplete. This is because each of the loops in the two interacting molecules is topologically equivalent to a circle (because of the base pairing at the cross-over point), and two circles, unlike two linear strands, are topologically unable to undergo the high degree of winding necessary to make a complete double helix. Some pairing is possible at the expense of twisting and tangling the rest of the molecule; the 'underwound loop' that results therefore contains both double-stranded and single stretches of DNA which are clearly visible in the electron microscope. Broker *et al.* use this method to reveal the short inverted repeats at the ends of the Mu G segment (presumably the G segment is a transposon since an identical sequence is present in phage P1, where it has much longer terminal inverted repeats), those at the ends of adenovirus-2, and at the two ends of the $\gamma\delta$ segment (a 5,700-base-pair sequence found at the boundary between F (sex factor) DNA and chromosomal DNA).

This technique will be valuable for detecting short inverted repeats separated by a spacer. I suspect that it will not be long before they are reported in eukaryote DNA. The fact that in *E. coli* the G segment can have either short terminal repeats (as in Mu) or long ones (as in P1) suggests that there is no fundamental functional difference between the long inverted repeats that abound in eukaryotes and the short ones which have so far been reported only in bacteria.

So far, ideas as to the function of palindromic sequences in eukaryotes are very vague (see *News and Views* **262**, 255; 1976). One possibility is that they have no function for the organism as a whole; they may even be disadvantageous 'selfish genes' maintained by strong positive selection at the level of the individual gene balanced by negative selection at the level of the organism; this would be entirely consistent with their tendency to trans-

The Sun and the weather

from Roger H. Olson

OVER the past few years, research into the relationships between solar activity (such as sunspots, flares and sector boundaries) and various climate and weather parameters has become more respectable. Although the search for mechanisms has as yet yielded few results, the number of impressive statistical correlations is steadily increasing. One of the best examples is the work of J. M. Mitchell, senior climatologist with the National Oceanic and Atmospheric Administration (NOAA). Mitchell has been looking at the correlation between sunspot activity and periods of drought in the Western United States, particularly since AD 1700, established from tree ring analysis.

Mitchell has worked with C. W. Stockton and D. M. Meko of the University of Arizona Tree Ring Laboratory, in studying the size of the area covered by drought in the Western United States. First, tree ring patterns, based on several types of trees, over the past 40 years were compared with well-known periods of drought. Once this standard correlation had been established it was possible to trace periods of drought back to AD 1700. A variety of sophisticated techniques were used to compare the period and geographical extent of the drought with the sunspot cycles, which have been observed reasonably consistently back to the beginning of the period.

No evidence was found for any influence of the 11-year cycle, but there is a very strong match between periods of drought and the 22-year double sunspot cycle (the Hale magnetic cycle). The area covered by drought tended to reach a maximum about 3 years after every other minimum in the 11-year cycle, with the most recent well-documented drought being that of the mid-1950s, correlat-

ing with the sunspot minimum of 1954. (The drought of the 1970s seems to be correlating with the sunspot minimum of 1976.)

The time between droughts averaged 20.4 years, whereas the average double sunspot cycle is 22 years. The reason for this apparent discrepancy is that the best coherence between drought area and sunspots comes during times when the sunspot maxima are high. It is during such periods that the time between successive maxima and minima tends to be smaller. When the sunspot numbers are small, the period of the cycle increases, and the correlation with drought area becomes weak.

Another tree ring series, dating back to AD 1600, was made available to Mitchell by H. C. Fritts. Although this series is not amenable to the statistical treatment mentioned earlier, it does contain one additional bit of information worthy of comment. During the Maunder minimum (see *News and Views* **236**, 405; 1977), when sunspots were virtually absent from the Sun during a 70-year period, the drought periodicity persisted in the Western United States. This suggests that the visible sunspot may not be the best parameter of solar activity to be used in the study of Sun-weather relationships.

A lesson to be learnt from Mitchell's work is that different meteorological parameters may behave in dramatically different ways. His study uses the area covered by drought, rather than the amount of rainfall or some other more arbitrary parameter. Rainfall statistics are notoriously hard to work with, because of their variability and dependence on topography. □

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locate and cause mutations. A more attractive proposition is that they may be the key to understanding the mechanism of cell determination. An important feature of determination is its great stability, inheritable over many cell generations. Although in principle this is explicable in terms of an association between DNA molecules and controlling proteins (and/or RNA) which is so stable that it can persist during replication and division, I think that the idea of a semi-permanent change in the DNA itself has been unduly neglected. Holliday and Pugh (*Science* **187**, 226; 1975) have put forward a most attractive mechanism involving

methylation of short palindromic sequences. Yet another possibility would be a change in the primary sequence of the DNA mediated by insertion sequences. Such a change would be inherited automatically by daughter cells, but would in principle be perfectly reversible (for example, when the nuclei of differentiated cells are transplanted into oocytes). Such changes could be inversions, deletions, duplications or translocations and could switch genes on and off by creating or destroying the recognition sites for controlling proteins. If the IS-mediated events commonly occurred only during DNA replication and were specifically

inhibited by the incorporation of bromodeoxyuridine into DNA then a jumping gene model could explain in a unifying way all the basic features of cell determination (see Reinert & Holtzer *Cell Cycle and Cell Differentiation*, Springer, 1975), including that in the immune system, as well as the apparent inconstancy in the position of palindromes in eukaryote DNA (Perlman *et al. Cell* **8**, 33; 1976). The trans-formed state in cancer (strongly heritable in somatic cells) could also result from a change in the primary sequence of the DNA concerned with the regulation of proliferation, as a result of stable aberrations in IS-mediated events (either spontaneous or mediated by radiation, chemicals or the insertion of viruses into the DNA).

Now that bromodeoxyuridine has been shown to inhibit bacterial sporulation (*Nature* **267**, 635; 1977) it may be possible to test such ideas in more tractable prokaryotic developmental systems. There may well be fewer basic differences between prokaryote and

eukaryote genetics than has hitherto been thought. The presence of long perfect palindromes in eukaryotes in spite of their absence in prokaryotes, for example, need not be a basic difference. It could be explained simply by supposing that IS sequences and transposons are even more important in eukaryotes than in prokaryotes and are so abundant that they are often adjacent; the junction between two adjacent transposons each of which has identical inverted repeats at its termini would be a perfect palindrome. Perfect palindromes would thus be merely the coincidental result of the coexistence in the genome of large numbers of transposons with similar termini.

Although a transposon or jumping gene theory of cell determination has many attractions, the key question about DNA palindromes is: will their study provide a way towards an eventual understanding of cell determination in eukaryotes, or shall we find that they only lead us back to where we started? □

discovered a receptor for adenosine which also activates adenylate cyclase. At this stage it seems that the adenosine and β -adrenergic receptors compete for the same enzyme, activating it through a common GTP-binding coupling unit. The adenosine receptor, however, seems to be coupled permanently to the enzyme. Further study of this system should be very instructive in sorting out the spectrum of receptor-adenylate cyclase interactions, including the possibility raised by P. Cuatrecasas (Wellcome Research Laboratories, Triangle Park) that the receptors reside permanently in separate membrane domains from the enzyme, never coupling but rather acting through molecular messages.

As the biochemists struggle to characterise a few receptors, so the pharmacologists keep increasing the choice for future biochemists. For example, β -adrenergic receptors in the brain as in peripheral tissue can be subdivided into β_1 and β_2 types (S. Nahorski, University of Leicester) and there seem to be several distinct classes of α -adrenergic receptor (D. H. Jenkinson, University College, London). Other new evidence on adrenergic receptors came in the Harden lecture delivered by S. H. Snyder (Johns Hopkins University). Both α and the β receptors in brain, he reported, are sensitive to sodium ions. Physiological concentrations of sodium decrease the binding of agonists but increase the binding of antagonists to the receptors (and the reverse effect can be demonstrated for the angiotensin receptor). That suggests a generalisation of the two-conformation receptor model previously proposed for the opiate receptor by Snyder.

Much pharmacological evidence for multiple opiate receptors (some recently published: *Nature* **267**, 577; 1977) was presented by H. Kosterlitz (University of Aberdeen). A new line of evidence comes from a comparison of the action of various opiate agonists in two strains of mice. Although enkephalin was equipotent in the two strains there was a large difference in their response to normorphine.

Most of the interest in the opiate receptor, however, remains firmly focused on the endogenous ligands. D. Smyth (National Institute for Medical Research, London) started with mention of the recently discovered very large '31K' precursor to both ACTH and β -lipotropin, the latter being the precursor of all the endogenous opiates except leucine enkephalin. He then summarised his (and others) evidence demonstrating high opiate-like activity of the peptide (C-fragment or β -endorphin) derived from residues 61 to 91 of β -lipotropin. C. R. Snell, from Smyth's

Receptors and hormones

by Peter Newmark

The Eleventh Harden Conference was held on 19–23 September, 1977 at Wye College. It was organised by Dr L. Iversen.

WHEN the identity of some receptors is about as substantial as the Emperor's new clothes it is always a real pleasure to hear of the nicotinic acetylcholine receptor. Even so there is still no purified receptor to show for the huge effort that has been made in that direction along a path strewn with discarded subunits.

Everybody finds a major acetylcholine-binding subunit of around 40,000 dalton by SDS electrophoresis but there is considerable dispute about the reality and significance of other, larger subunits. Are they part of the receptor (M. Raftery, California Institute of Technology), experimental artefacts (J. P. Changeux, Pasteur Institute) or simply absent (E. Barnard, Imperial College, London)? Barnard reported one of the first purifications of the acetylcholine receptor from mammalian tissue (denervated cat hind leg muscle) and found it to be remarkably similar to the more thoroughly studied receptors of the electric organs of the electric ray and eel.

A further growing problem in the

purification of the acetylcholine receptor is that of the functional heterogeneity of its subunits: it is not known how the binding of acetylcholine is translated into ionic transport across the membrane. Is this an integral property of the receptor itself or is a separate molecular entity responsible? Substantial evidence in favour of the latter was reported by M. Eldefrawi (University of Maryland) and by Changeux. They call the molecule the Ion Conductance Modulator or the Ionophore respectively.

The holy grail of receptor purification seems to be successful reconstitution of a purified, functional receptor in a synthetic lipid bilayer. Just how elusive that grail is, even for the acetylcholine receptor, was evident from the somewhat unseemly anxiety of all those present to point out the imperfections in all reconstitution data so far published.

Also on the slow but steady path to purification is the β -adrenergic receptor (A. Levitzki, Hebrew University of Jerusalem). At the same time Levitzki's group have accumulated kinetic evidence that the β -adrenergic receptor is not permanently coupled to adenylate cyclase. Instead a 'collision coupling' mechanism is involved in activation of the enzyme following ligand binding. That evidence comes from work on the turkey erythrocyte in which Levitzki has very recently

laboratory, next presented evidence for the existence of extracellular endopeptidases that could degrade β -endorphin to γ - and α -endorphins and to methionine enkephalin. He also showed that various experimental conditions for the extraction of endogenous brain opiates could favour activation of the degradative endopeptidases. Any suggestion however that such degradation accounted for the isolation of enkephalins rather than larger peptides from the brain was quickly discounted by Kosterlitz who pointed out that enkephalins have been extracted as the major brain opiates in conditions in which β -endorphin is not degraded.

Confirmation of that came from R. Miller (Wellcome Research Laboratories, Triangle Park) who then posed the question of the origin of brain enkephalin. The small amounts of β -lipotropin detected in the brain (Snell) indicate that enkephalin is metabolically derived from precursors synthesised *in situ*. Less likely is that brain enkephalin has its origins in β -endorphin released from the pituitary, especially since Miller showed that hypophysectomy had almost no effect on brain enkephalin content.

What then is the fate (purpose?) of the pituitary endorphin? The discovery of a hand-in-hand secretion of β -endorphin and ACTH into the bloodstream from the pituitary (*Science* 197, 1367; 1977) at least opens the possibility that it plays some part in the physiological response to stress.

Another group of peptide hormones to receive attention was the insulin family. T. Blundell (Birkbeck College, London) explained how it was possible to predict the basic three-dimensional structure of insulin growth factor (previously called non-suppressible insulin-like activity) and relaxin from their recently completed amino acid sequences and knowledge of insulin and proinsulin three-dimensional structure. When the structures are compared with that of insulin it becomes clear why insulin growth factor has insulin-like activity but relaxin has not.

Many of the remaining peptide hormones were encompassed in one grand scheme by L. Kohn (National Institutes of Health, Bethesda) who reviewed his evidence that the glycoprotein hormones are composed of two molecular subunits as is cholera toxin. One subunit is responsible for an initial interaction with a hormone-specific membrane ganglioside on the surface of target cells. As a consequence, the other subunit (characterised by a sequence homology that is present also in oxytocin and vasopressin) can penetrate the membrane, after which adenylate cyclase is activated. Recent evidence favours the

inclusion of tetanus toxin and interferon under the same mechanistic umbrella.

Kohn also reported evidence that one of the glycoprotein hormones, thyrotropin, causes an alteration in electrical potential across the membrane of cultured thyroid cells before stimulating adenylate cyclase (*Proc. natn. Acad. Sci. U.S.A.* 74, 2352; 1977). That led him to speculate that ion flow may be the primary event for all the ligands under his umbrella. Primary events were also the concern of R. Michell (University of Birmingham) who summarised the evidence that favours a crucial role for increased phosphatidylinositol turnover following ligand-receptor interaction in many non-cyclic AMP-dependent systems. Unfortunately the evidence, although substantial, is indirect and badly in need of direct, critical confirmation.

Finally a word in praise of the immunologists. They (M. Raff, University College, London; F. Melchers, Basel Institute for Immunology; H. Wigzell, University of Uppsala) superbly met the challenge of presenting their field to a largely ignorant audience in the space of an evening. □

Colonisation of a volcano inside a volcano

from Jared M. Diamond

THE regrowth of life on islands sterilised by volcanic explosions has offered biologists 'natural experiments' for studying colonisation. Classics of this type include studies of Krakatau (in Indonesia, exploded in 1883; Docters van Leeuwen *Ann. Jard. Botan. Buitenzorg*, 56-57, 1; 1936; Dammerman, *Verh. K. ned. Akad. Wet. Afd. Natuurk.* II, 44, 1; 1948; Borssum Waalkes, *Ann. Bogorieneses*, 4, 5; 1960) and Surtsey (off Iceland, arose from the sea in 1963; Fridriksson, *Surtsey*, Wiley, 1975). From observations of these islands, biologists have sought answers to questions such as: How do species differ in their ability to reach an empty island? Of the arriving species, who survives, and why?

A promising new study, by Eldon Ball (Australian National University), Joe Glucksman (Department of Primary Industry, Papua New Guinea), and Jean-Marie Bassot (CNRS, France) is of Long Island off the coast of New

Guinea. A recent visit adds to information that Ball and his coworkers gathered on five previous visits to Long since 1969 (Bassot & Ball *Papua New Guinea Sci. Soc. Proc.* 23, 26; 1972; Ball & Glucksman *Proc. R. Soc. Lond. B* 190, 421; 1975; Ball & Johnson in *Volcanism in Australia*, 133-147 (ed. R. W. Johnson), Elsevier, 1976; Ball *Australian Nat. Hist.* 19, 12; 1977). Factors making Long especially interesting are that its tropical location exposes it to a rich pool of colonists, that it is not just a single island but a nested set all of which have been at least partially defaunated in the past 300 years, that Ball's teams are virtually the only humans to have reached the innermost island (reducing the risk of species introductions by humans), and that there have been multiple colonisation episodes of this inner island associated with successive eruptions.

Long Island lies in the Bismarck Sea 30 miles northeast of New Guinea. It is a forested island of 170 square miles, in the centre of which is a crater lake (Lake Wisdom) of 37 square miles, in the centre of which is a small active volcano (Motmot Island) of about 10 acres, in the centre of which was (from 1968 to 1973) a small crater lake of about 1 acre. By a combination of biological, geological, historical and anthropological detective work, Ball has amassed evidence that an 18th-century explosion buried Long's former biota under tens of metres of lava. Lake Wisdom probably dates from that explosion, at least in its present form. The inner island, Motmot, is the tip of a submerged cone that erupted to form an island in 1943, was twice eroded down to the lake level and recreated by eruptions, and has existed continuously since 1968.

The outer island, Long itself, is already covered with rainforest. Its tree species are ones whose seeds arrived either by floating in the sea (for example *Terminalia catappa*, *Barringtonia* sp.), or else in the guts of birds (*Ficus*, *Canarium*, and *Eugenia* species, probably brought by fruit pigeons). In these forests now live nearly a full quota of bird species predicted for an island of Long's area (except for a deficit of montane bird species), as well as lizards (including the large *Varanus indicus*), snakes, bats, and the arboreal marsupial *Phalanger orientalis* (probably introduced by the humans who recolonised Long in the 19th century).

Lake Wisdom is remarkable for its depth of 1,200 foot, and for having high oxygen concentrations and aerobic invertebrate life down to the bottom. Most other large lakes in this region have anoxic bottoms lacking a bottom fauna. Two factors that may transport

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oxygen to the bottom of Lake Wisdom are wind circulation and convection currents caused by heating of the water around Motmot volcano. Although the lake's surface is at an elevation of 620 foot and the bottom is thus well below sea level, the water is nevertheless fresh down to the bottom, implying that the lake basin is sealed.

Lake Wisdom's biota is impoverished, compared with that of an older 'control' lake nearby (Lake Dakataua), probably because the lake is little more than two centuries old. The most striking absences are of vascular aquatic plants, copepods, fish, and turtles. However, there are many species of benthic algae, invertebrates (aquatic insects, molluscs, crustaceans, and one species of sponge), and one species each of nesting duck and grebe. Winds and birds probably brought the plants and invertebrates. But the lake also contains at least one adult crocodile. Crocodiles periodically reach the sea-coast of Long in floating rafts of vegetation from New Guinea. How they got several miles inland and up and over the steep crater rim into the lake remains a mystery.

The inner island, Motmot, is difficult of human access because of the steep crater rim, lack of human population on Lake Wisdom's shore, the four miles' distance over the wind-tossed lake from the shore, crocodiles in the lake, and near-boiling water temperatures on some sides. Ball and his co-workers have variously reached Motmot from the lake shore by native-built outrigger canoe, collapsible aluminium boat, dinghy, or swimming while pushing a raft and avoiding the areas of near-boiling water. On Ball's first visit in 1969, 1 year after recreation of Motmot by an eruption, the sole mature plant was a sedge, the sole land invertebrate a lycosid spider. Over the next 4 years the number of plant and invertebrate species respectively increased to 17 and 20, until a new eruption in 1973 destroyed the inner crater lake and wiped out most plants except a sedge. Ducks and swallows nest on Motmot, migrant shorebirds from the northern hemisphere periodically visit, and a falcon visits from Long itself to devour its prey of small birds.

How do the plant and invertebrate colonists reach Motmot? Some arrive wind-blown by aerial rafting. Many are washed up on Motmot's beach, having floated from Long across Lake Wisdom by themselves or on floating logs. Ball found five species of invertebrates on a log that had floated past Motmot. Numerous seeds that wash onto the beach sprout but fail to survive the combination of heavy wave erosion and changing water levels. Some seeds come attached to the feathers and feet of the

nesting ducks and grow mainly in the areas that the ducks frequent. Still other seeds arrive in the guts of the falcon's bird prey.

What invertebrates colonise Motmot, where do they live, and what do they eat? On the beach are beetles, earwigs, ants, and bugs, living off washed-up organic material. Under an algal crust on the inner crater lake are collembolans and rove beetles. Around this lake are ants, scavenging or eating smaller insects. It was a surprise to find a lycosid spider as the earliest land colonist, since they are strict carnivores and since scavengers dominated the early colonists of Krakatau and Surtsey (but a spider was the first organism found on Krakatau after it exploded). What would a solitary carnivore eat? Probably aerial plankton; but lycosids can gorge themselves and survive half-a-year without food.

As yet, virtually no attention has been paid to comparing the species composition of Long's flora and insect fauna with the source biota of New Guinea and New Britain. This would be a rich data base for understanding who does and does not colonise. It could also suggest how much time is necessary for genetic divergence and subspecies formation. For example, the description of an endemic bird subspecies from Long (Salomonsen *Breviora Mus. Comp. Zool. Harvard*, no. 254; 1966) suggests formation of a bird subspecies within two centuries. □

Tibetan meteorology

from T. B. Tang

In its working conference held in June, the Academia Sinica confirmed that weather and long-term climatic forecasting has been selected as a key research topic in China; in the same month, its Institute of Atmospheric Physics launched a new journal—*Atmospheric Science*—devoted principally to climatic research. (From the second issue onwards this journal is available and has a table of contents in English). Meteorologically, the Chinghai-Tibet Plateau in western China is of immediate relevance, if only because of its vastness (one-fourth of China's land area). The Plateau has immense dynamic effects on the atmospheric circulation in the Northern Hemisphere and constitutes an important influence on the weather and climate of areas eastward as far as the Japanese Islands.

Recently, there has been much

scientific activity in Tibet. For instance, exploiting the high altitude, the Chinese have set up there an observational station for cosmic rays. Hard on the heels of the completion of field work for the comprehensive geological and biological survey (see *News and Views* 269, 12; 1977), the first major meteorological programme commenced in July this year and, after three months, has now come to a close (*Hsinhua News*, 10 October).

The research programme dealt with weather patterns on the Plateau in the summer, and was carried out collectively by personnel of the Central Meteorological Bureau, of provincial bureaux in northwest and southwest China and the Army, as well as by local meteorologists.

The work included the study of meteorological data collected between 1969 and 1976, the analysis of cloud photographs taken by satellites in recent years, and field surveys along the middle reaches of Yalu-tsaupo River. The detailed findings are not yet available. However, the general picture obtained is that in summer, a high pressure air mass forms in the upper level of the atmosphere; when it moves eastward, it causes drought in the other parts of China. At low levels in the atmosphere there also frequently occur low-pressure eddies and shear lines. These are the weather systems responsible for precipitation in summer on the plateau, bringing damaging heavy rain. In certain circumstances they move eastward and cause devastating floods in southwest, central, and eastern inland China. An understanding of the formation, evolution, and distribution of these eddies and shear lines, as well as their directions of movement and seasonal variations, and the relationship between these factors and precipitation is, therefore, vitally important to weather forecasting and the mitigation of natural calamities.

In the present connection it is of interest to note a review article discussing climatic changes in the Plateau for the past 500 years, which appeared in the September 1977 issue of the popular science journal *Ke Xue Shi Yan*. The article reported that an

Correction

In the article 'Carbon fixation pathways' (*News and Views* 269, 201; 1977) paragraph 5, line 7 and following should read "Measurements of leaves of quantum yield for CO₂ uptake show the following. At normal oxygen tension and 30 °C, C₃ and C₄ plants show similar efficiencies, but as CO₂ tension increases the efficiency of C₃ metabolism increases whereas C₄ remains constant."

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annual mean temperature profile has been worked out, using evidence from the analysis of the annual growth rings of trees; samples were selected from trees found at around 4,300 m above sea level. On the other hand, trees growing at about 3,000 m on forest edges, where moisture rather than warmth becomes growth-limiting were used for estimating annual precipitation. In spite of greater variations from place to place, the picture has emerged that in the 1770s, the 1830s and the early 20th century, annual precipitation was on the whole higher than that at present. It was lower, however, during the longer periods of the late 18th century, the second half of the 19th century, and the mid-20th century. In the past 20 years rainfall has been steadily decreasing by a relatively large total amplitude of approximately 10%. It has been possible to check this picture independently with the observed history of water levels in lakes (from field surveys or satellite pictures), the motion of glaciers (especially their regression), the movements of snow-lines as well as water table levels, and in a few cases with documented records. In general, the results are all consistent with the proposed former variations in annual mean temperature and precipitation. □

Singlet oxygen in chemistry and biology

from R. B. Cundall

An International Conference on Singlet Oxygen and Related Species in Chemistry and Biology was held on 21–26 August, 1977 at the Whiteshell Nuclear Research Establishment in Pinawa, Manitoba. Many of the papers will be published in a special issue of *Photochemistry and Photobiology*.

THE Earth has an atmosphere containing diatomic triplet oxygen ($^3\Sigma_g^-$); essential for life in the form in which we know it. Consequently an understanding of the chemistry of all oxidation processes which have both beneficial and harmful effects is one of the major problems confronting both biochemists and chemists. The meeting aimed to foster the interaction of physical chemistry with biology in elucidating oxidative transformations, and attracted scientists from fields as diverse as medicine and molecular physics.

A feature of oxygen chemistry is

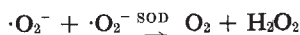
the interconvertibility of reactive oxidising transients. Attention was mainly directed to the behaviour of $^1\Delta_g\text{O}_2$, $^1\Sigma_g^+\text{O}_2$, $\cdot\text{OH}$, $\cdot\text{O}_2^-/\text{HO}_2\cdot$, O_3 , H_2O_2 , organic peroxides and hydroperoxides, and derived species such as some of the oxidising inorganic radical anions. Different groups of specialists, showing common interests in oxidation mechanisms presented new work using a wide range of techniques. Particularly prominent were laser photolysis, pulse radiolysis, luminescence, chemiluminescence, ESR, NMR, as well as conventional biochemical techniques, steady state photolysis and radiolysis with product analysis.

Quantitative measurements, mainly by pulse radiolysis, show hydroxyl radicals to be very reactive and to interact unselectively with most biological molecules at rates which are practically diffusion controlled. The fate of the secondary radicals formed by attack on substrates was very much a subject for discussion since it is clear that oxidation in biochemical systems must generate a species of reactivity comparable to OH. The process usually cited to explain this has been



a component of the now classical Haber-Weiss mechanism (1934). This cannot be the reaction involved since it has been established as slow (B. H. J. Bielski, Brookhaven National Laboratory; G. Czapski, Hebrew University, Jerusalem). Alternatives proposed were metal-mediated processes, enzyme-metal catalysis or involvement of peroxides, particularly those derived from lipids.

The low reactivity of the $\cdot\text{O}_2^-/\text{HO}_2\cdot$ system is now widely agreed and it is clear that in general $\cdot\text{O}_2^-$, like H_2O_2 , reacts only with such readily reducible molecules as those containing labile SH groups. Nevertheless the biochemical role and significance of the superoxide anion is puzzling. The xanthine oxidase enzyme system is an efficient O_2^- generator. The superoxide dismutase (SOD) whose function was first defined by one of the participants, I. Fridovich (Duke University, North Carolina), apparently exists solely to bring about



The nature of this type of enzyme and its properties was a dominant topic in both presentations and discussion. The pulse radiolysis technique has been particularly successful in elucidating the mechanism of these metallo-enzymes (E. M. Fielden, Institute of Cancer Research, Sutton, Surrey). The

use of catalase to remove H_2O_2 completes an enzyme trio used to a large extent in mechanistic studies.

Radiation chemistry now has an established role as the most prolific source of information on free radical reactions in aqueous solution and several papers gave evidence of progress in the area. A significant contribution to radiobiology was the demonstration that SOD is an effective radioprotector exerting a protective role even when administered after radiation doses have been delivered (A. Petkau, Whiteshell Nuclear Research Establishment). This clearly shows where knowledge of primary radiation effects and oxidative enzyme reactions can be combined to advantage.

The role of $^1\text{O}_2$ in biochemical systems is not yet clear. It does not seem to be formed in significant yields from O_2^- , in polar systems at least, although it can be formed by chemical reactions which generate it directly or by energy transfer to $^3\Sigma_g^-\text{O}_2$ from a product formed in the triplet state. The paper of J. Cadet (Centre d'Etudes Nucleaire de Grenoble) in which methodical attempts to generate separately $^1\Delta_g\text{O}_2$ as well as OH and O_2^- and then examine their reaction with DNA constituents shows how the subject is likely to progress in the immediate future.

The use of dye photosensitisers in research on the so-called photodynamic effect is an area in which an important role for $^1\Delta_g\text{O}_2$ is beyond dispute. The complications arising in particular cases from the simultaneous participation of the Type I (free radical) and Type II ($^1\Delta_g$) processes were described by several authors and the behaviour related to dye and binding properties (T. Ito, University of Tokyo). A number of papers dealt with experimental techniques, especially interesting were those on the use of dye lasers for excitation of singlet oxygen.

Many interesting studies on specific systems were reported; for example M. Kasha (Florida State University) has found that pressurisation by oxygen produces complexes which remain even when the pressure is reduced. The $^3\text{E}_{1u}$ state of benzene has been revealed and the $\text{T}_1 \rightarrow \text{S}_0$ emission from pyridine seen for the first time by this method.

An experiment in conference organisation was a public meeting held on the first evening in which five speakers gave short presentations of interest relevant to the content of the scientific programme to a wide audience. Topics were skin sensitivity to light (M. M. Mathews-Roth, Harvard Medical School), free radicals and ageing (J. D. Crapo, Duke University, North Carolina), sunlight and air pollution (B. D. Goldstein, New York University), the ozone layer (H. R. Rawls,

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Louisiana State University), and the fate of spilled oil (R. A. Larson, Stroud Water Research Centre, Avondale, Pa.). More than 300 people from a small community attended and there was active questioning and discussion. This might be a possible way for scientists to bring their scientific work and opinions directly, rather than through the media, to a lay audience, especially on controversial topics. □

Thin film technology

from C. W. Pitt

The first of an intended series of international conferences on ion-assisted thin film coating techniques was held in Edinburgh on 8–10 June 1977. The Proceedings are available from CEP Consultants, Edinburgh. The next IPAT conference will be held in London in July 1979.

THE presentations covered a wide range of experimental and production processes, ranging from ion plating, ionised clusterbeam technology, r.f. sputtering, magnetron sputtering, sputter-ion plating, ion implantation and activated reactive evaporation and plasma deposition. An equally large range of applications of the various methods was presented including tribology, tool coatings, corrosion resistant turbine and airframe components, decorative finishes, gas storage, semiconductor, optical and solar devices, electronic components and steel hardening.

A number of the papers were of particular interest to electrical and electronic engineers and solid-state physicists. The paper by T. Takagi *et al.* (Kyoto University) on ionised cluster-beam technology suggested that the required incoming material mobility on the substrate can be achieved by partially ionising clusters of atoms from an adiabatic expansion source and, provided that the deposition rate is maintained at least 10^3 higher than the residual gas impingement rate, single crystal and near bulk density films of a wide range of materials may be deposited. The technique has been applied in numerous ways—for example, epitaxial silicon on silicon, InSb on sapphire, GaAs on Cr: GaAs. Silicon solar cells grown n on p, have been fabricated with exposed surface-layers of 140 Å and which exhibit 70% of the peak output voltage from 450 nm to 950 nm. Low resistance noble metal films deposited on glass substrates have

demonstrated near bulk resistivity in films 200 Å thick; thicker films of the same metals have been used as interconnections on passivated semiconductor chips and have successfully covered 8 µm oxide steps. Furthermore, ohmic contacts have been made to semiconducting material (Ag on n-type silicon, AuBe on p-type GaP) without interface metals or sintering. Ionised cluster-beam deposition on Mn-doped ZnS was also reported to have produced d.c. electroluminescent displays of similar properties to those produced by evaporation and subsequent implantation of the dopant. The superior adhesion properties of the process were demonstrated by the fabrication of flexible printed circuit board which meets the requirements of IPC-FC-240B specification.

K. Jones *et al.* (ICI, Runcorn) have also examined Au, Cu, and Al layers deposited on several types of flexible plastic substrates—polyethersulphone, polypropylene, polyimide and polyester. They concluded that the Cu: polyethersulphone system was particularly suited for flexible pcb construction by the ion-plating process. Ion plating produced good decorative finishes for most of the metal/plastic combination. Sims, in a review of ion-plating applications, again mentioned the fabrication of printed circuit board material. He also cited a number of less obvious applications such as battery-grid coatings which result in an improvement in the energy storage efficiency of lead/acid cells, aluminium coatings for steel and plastic lamp-reflectors and which are subsequently protected by ion-plated glassy layers, and also coatings for X-ray anodes for use in the soft wavelength spectrum. B. Heinz (Leybold Heraeus, Hanau) reported the use of electron-beam ion plating for forming the contact layers of noble metals on reed-relay blades. He and his colleagues have found that the switching lifetime of the plated blades could be enhanced by a factor of 10 times in appropriate conditions, and that the process produced blades with reduced sticking probability and contact resistance. The process is in industrial production.

Anti-reflective and protective coatings for germanium lenses for infrared imaging were discussed by E. Henderson (Pilkington, St Asaph). Although the experiments reported were in the early stages, it was clear that serious consideration is being given to the use of ion plating for depositing ZnS anti-reflection coatings on five-element large-area zoom lenses for operation at 8–12 µm in corrosive atmospheric conditions, in aircraft (for example). Preliminary data indicate an improvement in lifetime of four to eight times in

adverse conditions. C. W. Pitt (University College, London) reviewed the r.f. sputtering process, with particular emphasis on the capability of this process for depositing dielectric films with good optical properties. The technique has been found to be attractive for fabricating optical waveguiding components for integrated-optics applications. Hard graphitic carbon deposits may be prepared by vapour deposition from a hydrocarbon gas excited into a plasma-phase by an r.f. field. S. M. Ojha (University of Sussex) presented some details of this process and of the films produced—it was projected that the rather impressive hardness (unscratched by tungsten carbide) and chemical passivity (insoluble in any of the solvents and acids used, including hydrofluoric acid) might lead to applications in semiconductor passivation and infrared filters.

The increasing use of nuclear power stations for generating electricity has produced several contentious issues, not least of which is the disposal of radioactive wastes. The storage of active gases is particularly sensitive in view of the rapid dispersal if the container is damaged. D. S. Whitmell *et al.* (UK Atomic Energy Authority) have devised an ingenious method of containing radioactive ^{85}Kr for the required 100–200 yr and which greatly reduces the potential danger if the container is damaged. The gas is incorporated into a copper thin film matrix by plating a thin film of the metal on a substrate by d.c. sputtering, followed by a reversal of the field so that gas ions are bombarded at the deposited film, penetrate and are trapped. The process is then repeated many times until a thick deposited layer with occluded gas entrapment is built up. It was claimed that up to 5% (atomic) gas occlusion could be achieved—a similar storage capability to a high pressure gas cylinder (approximately 170 l of gas at NTP per l of metal). Damage to the deposit results in very little gas release and only from the area local to the damage.

Two papers on ion implantation, by C. Dearnley *et al.* (Atomic Energy Research Establishment, Harwell) and by V. Ashworth *et al.* (University of Manchester Institute of Science and Technology) suggest that semiconductor devices are not the only area in which the electronic/electrical industry will be influenced by this process. The papers primarily dealt with ion implantation of dopants into metals as a means of altering the wear-resistance of the host metal. But it was apparent from the results of tests on the electrochemical potentials of the doped materials that cathodic protection with improved efficiency may well be feasible. □

review article

Biochemistry of the bacterial catabolism of aromatic compounds in anaerobic environments

W. Charles Evans*

*Methods of aerobic degradation of aromatic compounds in the biosphere are well understood, but it is only relatively recently that it has been shown how some bacteria can also degrade these substrates in the absence of molecular oxygen. This occurs by photometabolism (*Athiorhodaceae*), nitrate respiration (*Pseudomonas* and *Moraxella* sp.) and methanogenic fermentation (a consortium) in which the benzene nucleus is first reduced and then cleaved by hydrolysis to yield aliphatic acids for cell growth. These methods may be used by microbial communities to catabolise man-made pollutants.*

THE biogeochemical cycles which operate on Earth are essential for the maintenance and continuation of life as we know it. Solar radiation provides the energy which is harnessed by photosynthesis. Relatively simple inorganic chemicals (supplied by soil minerals, natural waters and air) are utilised by autotrophs for their biochemical processes; these feed the heterotrophs. After death, the constituents of living organisms are mineralised again by microorganisms. This dynamic sequence of events illustrated by the biological carbon cycle (Fig. 1) has evolved over a geological time scale and in a variety of physical conditions.

Although there is diversity and increased biochemical complexity as we pass from bacteria to plants and animals, their cellular metabolism and the structures concerned with the basic reactions of life have much in common. Yet, their biosynthetic and degradative powers differ markedly. Plants are unique in making large quantities of insoluble polymers, including the aromatic macromolecules—lignins and tannins—which only microorganisms can degrade. This sector of the C cycle shown in Fig. 2 has the slowest turnover rate of all natural products¹; soil humus, however, confers beneficial properties on this medium for plant growth.

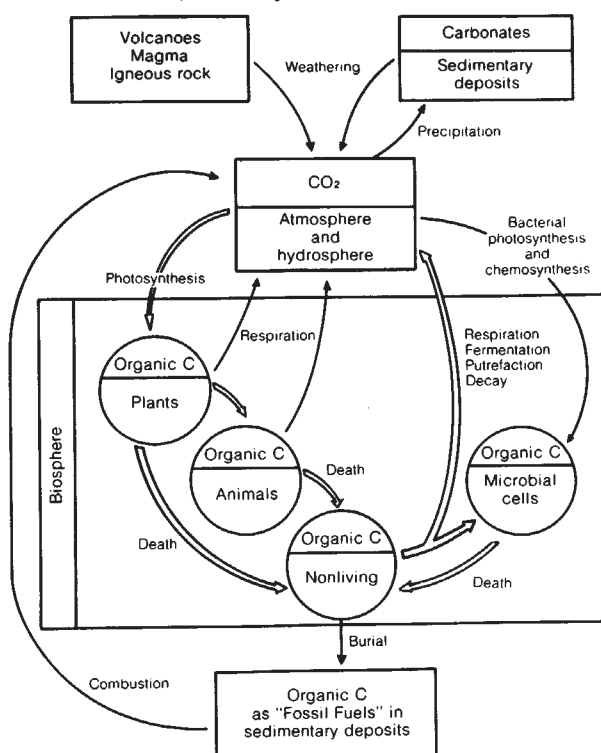
Microorganisms have evolved enzymes of impressive versatility to metabolise natural products; use is made of these in the food industries, water purification and sewage treatment. Science is also applied to control other sectors of the biological C cycle, for example, artificial fertilisers, herbicides, fungicides and insecticides to increase food production; detergents and pharmaceuticals for health reasons. Many of these are synthetic, xenobiotic, aromatic organic chemicals and together with the ever increasing waste products of industry, present an added burden for the mixed microbial populations of soils and natural waters to dissimilate.

Three general methods exist for the catabolism of organic compounds. (1) In aerobic respiration, the oxidation stages occur at the expense of molecular oxygen as the terminal electron acceptor; O_2 is also a reactant for oxygenase enzymes since it is incorporated into the products. The availability of O_2 is often a limiting factor in mineralisation. (2) In anaerobic respiration, bacteria use inorganic electron acceptors to metabolise substrates in the absence of air. Thus, NO_3^- is reduced to N_2 or NH_3 ; SO_4^{2-} to S^{2-} ; and CO_2 to methane (CH_4). Although pathways of degradation are usually identical in aerobic and anaerobic respiration, this is not the case for aromatic compounds. (3) In

fermentation, no external electron acceptor is required, the carbon source is degraded anaerobically by a series of reactions that release energy by substrate-level phosphorylation. There is a wide range of fermentation products; aerobic processes are necessary to complete that part of the carbon cycle initiated this way.

Clearly, the utilisation of any of these pathways is determined by the nature of the habitat. Aerobic and anaerobic environments exist in the biosphere; if the oceans are taken into consideration, the size of the latter far exceeds that of the former². Conditions must also be such as to sustain microbial life—nutrients must be available and the pH and temperature favourable—for mineralisation to occur. Vast quantities of organic matter was seques-

Fig. 1 The biological carbon cycle. (Modified from original in *Introduction to Bacteria and their Ecobiology*, by R. N. Doetsch & T. M. Cook, University Park Press, Baltimore, 1973).



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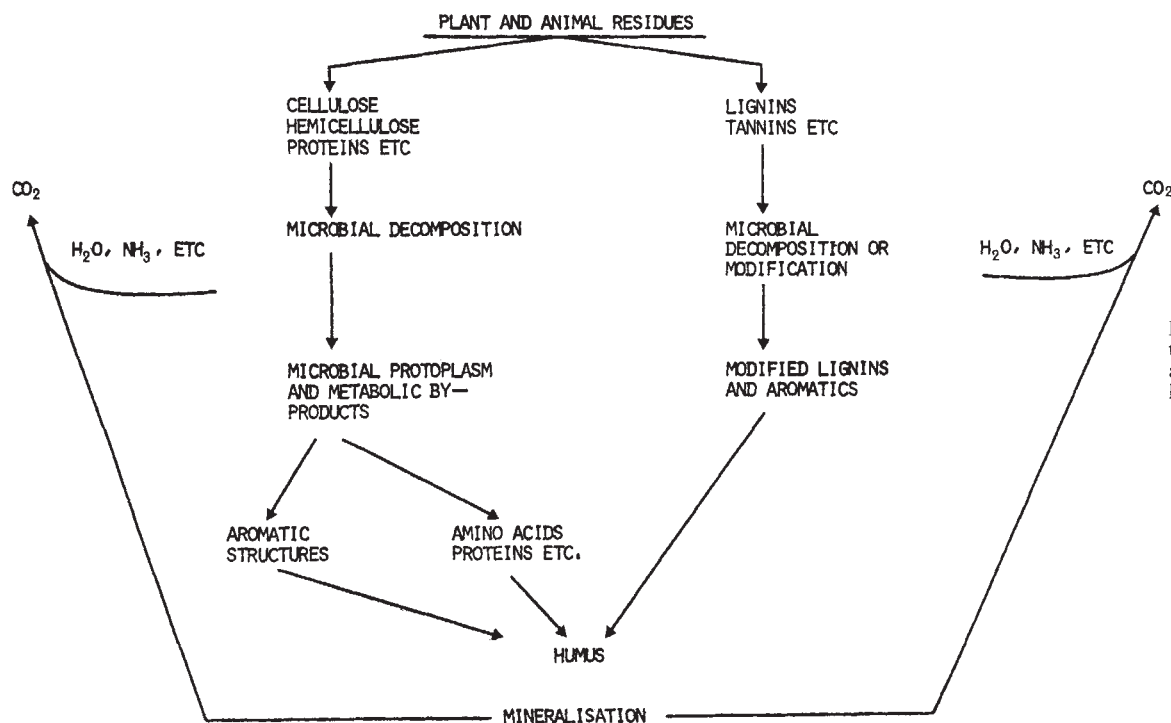


Fig. 2 Organic matter decomposition and formation of humic substances in soils.

trated in the lithosphere in ancient times—now forming the coal, oil and natural gas measures—which remained virtually intact until man began to bring these sources of carbon and energy into circulation again. Soils and sediments provide all the conditions mentioned above; the continuous alternation between aerobic and anaerobic habitats control the biological carbon, nitrogen, sulphur and other cycles in soils³.

These factors are well illustrated when we consider the various methods of microbial degradation of aromatic compounds in nature; they also have a basic relevance to the problems of environmental pollution.

Catabolism of aromatic compounds

It is about 30 years since the salient chemical features of the aerobic *ortho* (intradiol) pathway of bacterial aromatic-ring metabolism was elucidated⁴⁻⁸. This was followed by the discovery of the alternative *meta* (extradiol) method by which some bacteria cleave the benzene nucleus⁹⁻¹². These aerobic pathways are initiated by microbial mono- and di-oxygenases; molecular oxygen is essential for them to function since it is incorporated into the reaction-products¹³. They have been extensively reviewed¹⁴⁻¹⁶ and need no repetition here.

Natural selection among microorganisms has explored the chemical possibilities of how to convert stable aromatic structures into useful metabolites; all aerobes employ the device of oxygenative ring-cleavage. In anaerobic conditions this is prohibited; evolutionary considerations indicate that the only remaining options available to bacteria in the primitive biosphere with its anoxygenic atmosphere are either hydration or hydrogenation followed by non-oxidative ring fission. Chemical considerations indicate that for additions to an aromatic nucleus to occur, the system of bonding characterised by extensive delocalisation of the Π electrons must be converted into one in which little of the delocalisation energy remains. Hydrogen saturates the aromatic ring under moderate conditions, whereas addition of water is not observed and is thermodynamically improbable. Predictions made on chemical reactivity alone are not necessarily valid for enzyme-catalysed reactions; indeed, both ideas were at one time entertained. It has now been established that in all cases investigated where aromatic substrates are degraded under anaerobic conditions, the microorganisms utilise the remarkable biochemical device of initial ring reduction, first announced by Dutton and Evans¹⁷.

Indications that anaerobic methods of aromatic-ring met-

abolism exist in nature which permit cleavage without the participation of oxygen *per se* was provided by Tarvin and Buswell¹⁸. They reported that benzoate, phenylacetate, phenylpropionate and cinnamate were completely utilised with the production of CO₂ and methane by a sewage-sludge inoculum in strictly anaerobic conditions. During the past decade advances have been made in understanding the biochemistry of this phenomenon. It transpires that the anaerobic dissimilation of the benzene nucleus occurs in at least three different sets of biological conditions and it is the main purpose of this article to delineate progress made in each case.

Anaerobic photometabolism of benzoate by the Athiorhodaceae

Several species of the purple non-sulphur bacteria, the *Rhodospirillaceae* are able to grow at the expense of simple aromatic compounds as sole carbon source both anaerobically in the light by photosynthetic means and aerobically in the dark by respiration. Proctor and Scher¹⁹, studying the photometabolism of benzoate by *Rhodopseudomonas palustris*, assumed that the 'bound oxygen' produced by the light reaction (van Niel²⁰) was equivalent to molecular oxygen; a pathway similar to the known aerobic methods of dissimilation was therefore suggested. Leadbetter and Hawk²¹, and Dutton and Evans²², however, obtained results which made this hypothesis untenable; *Rhodopseudomonas palustris* cells grown photosynthetically on benzoate or the hydroxybenzoates showed no respiratory activity with these substrates in aerobic conditions. Furthermore, such cells were devoid of the enzymes of the aerobic pathways, and the familiar intermediates were not detectable in culture. Figure 3 illustrates dramatically the inhibitory effect of oxygen and the obligatory requirement for light in the photometabolism of benzoate.

It became apparent that another method of biochemical attack on the benzene nucleus must occur in these conditions; the addition of hydrogen seemed to us a distinct possibility. Unlabelled 'test' intermediates to be expected if a reductive pathway operated, were incubated with cell suspensions of *Rhodopseudomonas palustris* actively photometabolising (U-¹⁴C) benzoate (I); after appropriate time intervals the test compounds were extracted and examined for any isotope exchange. Cyclohexanecarboxylate (II), cyclohex-1-enecarboxylate (III), 2-hydroxycyclohexanecarboxylate (IV), 2-oxocyclohexanecarboxylate (V), and pimelate (VI) became labelled—indicating that the seven carbon atoms of benzoate remain together until after the

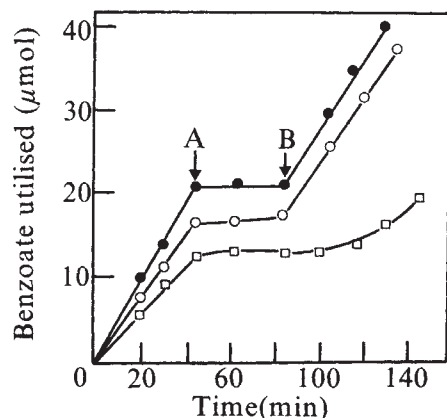


Fig. 3 The effects of air, light and darkness on the photometabolism of Benzoate by *Rhodospseudomonas palustris* cells. The initial conditions were anaerobic with illumination. In the period (A-B) between the arrows the conditions were: air-light (○), air-dark (●) and anaerobic-dark (□).

ring cleavage stage. These results suggested^{17,23} a series of reactions involving the reduction of benzoate (or a derivative) to a cyclohexanecarboxylate moiety followed by a coenzyme-A mediated β -oxidation sequence. Because the reduced acid is alicyclic, instead of the usual release of acetyl-CoA, breakage of the bond in the 1,2 position occurs to yield pimelate (or its equivalent). This new reductive pathway illustrated in Fig. 4 subsequently received independent support from Guyer and Hegeman²⁴ who showed that the behaviour of *Rhodospseudomonas palustris* mutants was consistent with such a scheme. We have recently demonstrated the occurrence of these reactions using sub-cellular fractions from *Rhodospseudomonas palustris* grown photosynthetically on benzoate with the following results (ref. 25 and D. O. Lunt and W.C.E., unpublished). (I) A washed chromatophore suspension reduced benzoylphosphate but not benzoate or benzoyl-CoA; light and anaerobic conditions were essential for this to occur. A cell-free extract in the presence of CoA, ATP, NAD⁺ and Mg²⁺ ions converted cyclohex-1-enecarboxylate to pimelate in anaerobic

or aerobic conditions and in the light or dark.

These results provide strong evidence for the presence of a light-dependent membrane bound proton-translocating redox system in these chromatophores; the low potential reductant may be a ferredoxin, which also plays a part in photosynthetic electron transport. In addition, they confirm the presence of the appropriate β -oxidation suite of enzymes in these cells responsible for the subsequent series of reactions resulting in ring-cleavage.

Although all members of the Athiorhodaceae examined utilise several aromatic acids photosynthetically, some can also metabolise phloroglucinol in these conditions. Using *Rhodospseudomonas gelatinosa*, we detected dihydrophloroglucinol and 2-oxo-4-hydroxyadipate in photosynthetic cultures growing on phloroglucinol as sole carbon source²⁵. Surprisingly, a soluble extract from these cells reduced phloroglucinol to dihydrophloroglucinol in the presence of NADPH as specific hydrogen donor in the dark. Our knowledge of the subsequent events leading to ring-fission is at present fragmentary, but is tentatively formulated as in Fig. 5.

Anaerobic metabolism of benzoate through 'nitrate respiration'

Oshima²⁶ described a bacterial culture containing two different organisms from soil, which in combination but not separately, grew anaerobically on a variety of aromatic substrates in the obligatory presence of nitrate. Using N¹⁸O₃ and *p*-hydroxybenzoate or protocatechuate as substrate, these organisms incorporated more of the heavy oxygen isotope than with succinate as the C source. He concluded that in the anaerobic cleavage of the aromatic ring, the oxygen atoms of NO₃ were used in a manner similar to that of molecular oxygen. This definitive inference seems unwarranted, bearing in mind the nature of his experiments. Taylor and his collaborators^{27,28} then isolated from soil a pure culture of a *Pseudomonas* strain (PN 1) with a similar nitrate-dependent anaerobic metabolism of aromatic acids. Their results showed quite clearly that the anaerobic breakdown of the aromatic ring is different and quite distinct from the aerobic pathways. Although unsuccessful in revealing the pathway, they suggested that aromaticity was destroyed by the addition of three molecules of water to the benzene nucleus in preparation for ring

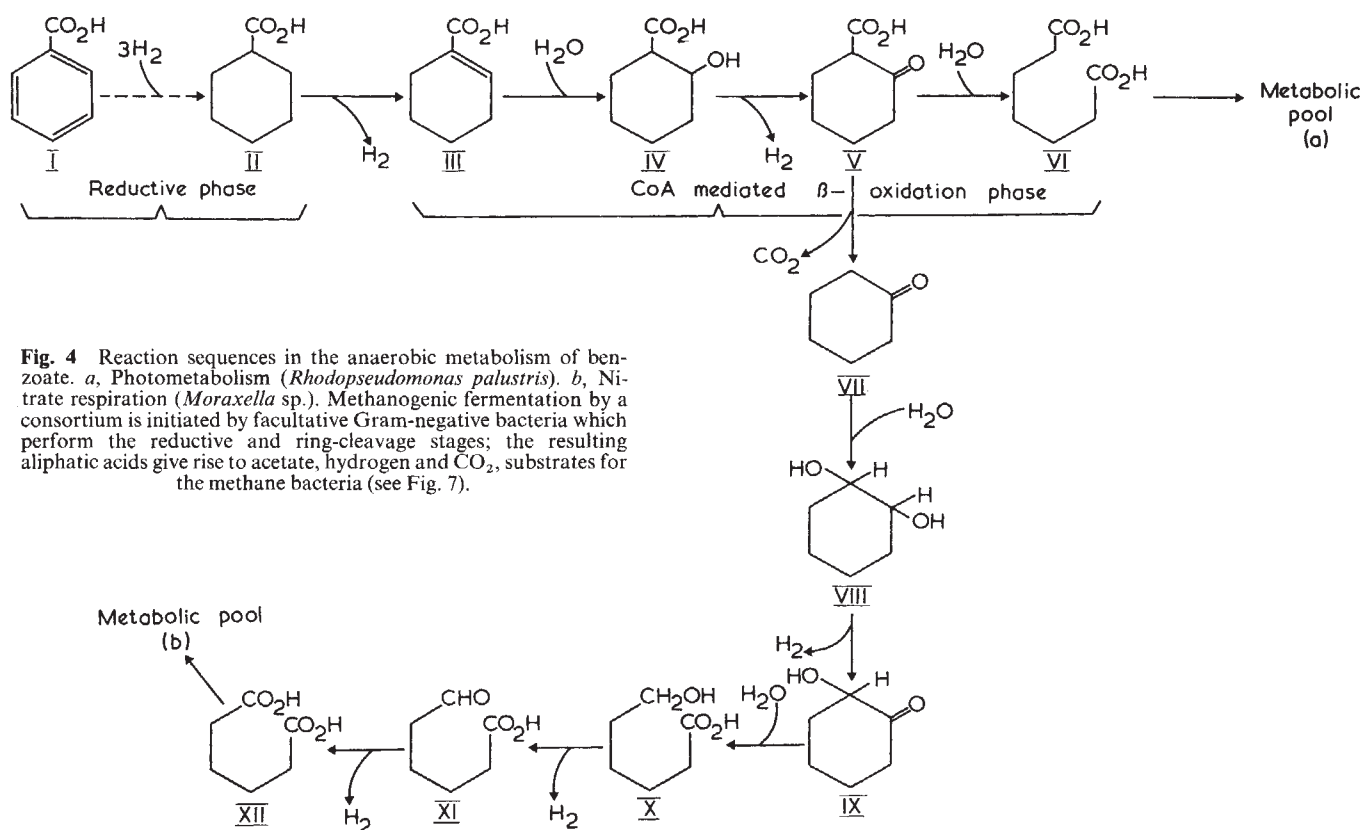


Fig. 4 Reaction sequences in the anaerobic metabolism of benzoate. a, Photometabolism (*Rhodospseudomonas palustris*). b, Nitrate respiration (*Moraxella* sp.). c, Methanogenic fermentation by a consortium is initiated by facultative Gram-negative bacteria which perform the reductive and ring-cleavage stages; the resulting aliphatic acids give rise to acetate, hydrogen and CO₂, substrates for the methane bacteria (see Fig. 7).

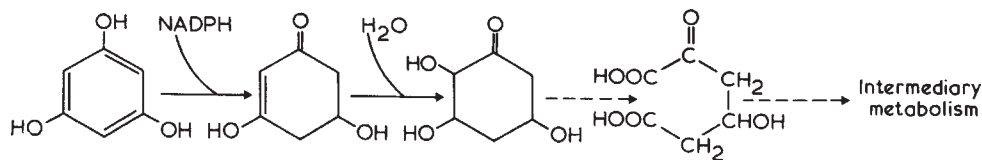


Fig. 5 Anaerobic metabolism of phenol by *Rhodospseudomonas gelatinosa*.

cleavage. (A similar mechanism had been suggested for the aerobic catabolism of aromatic substrates²⁹.) We could not agree with their hypothesis on theoretical grounds, yet these workers had drawn attention to an important phenomenon. We also isolated a Gram-negative bacterium from soil which metabolised benzoate anaerobically in the presence of nitrate^{30,31}; it turned out to be a *Moraxella* sp. (N.C.I.B.11086) instead of a *Pseudomonas*. Concomitant with the disappearance of the aromatic substrate in these *Moraxella* cultures, nitrate was reduced to nitrogen gas. When (ring-¹⁴C) benzoate was incubated anaerobically with these cells in nitrate-phosphate buffer, we identified labelled cyclohexanecarboxylate (II), cyclohex-1-enecarboxylate (III), 2-hydroxycyclohexanecarboxylate (IV) and adipate (XII). Using (carboxy-¹⁴C) benzoate in a similar experiment, the above intermediates were again radioactive with the exception of adipate. Subsequently, we were able to show that *Pseudomonas* PN1 (obtained through the courtesy of Dr Taylor) behaved similarly.

These results imply that the reductive pathway operates in the anaerobic catabolism of aromatic substrates through nitrate respiration³¹. The production of adipate, instead of pimelate in these cultures is explained by a divergence at the 2-oxocyclohexanecarboxylate stage, with its decarboxylation to cyclohexanone (VII) followed by alicyclic ring-cleavage by an unknown mechanism, as shown in Fig. 4.

Recently, Bakker³² has demonstrated that a mixed bacterial culture adapted from a primary inoculum consisting of a mixture of soil, manure and sewage-sludge, degrades phenol in anaerobic conditions in a nitrate-mineral salts medium. The qualitative composition of this consortium remained fairly constant on serial transfer in liquid culture and consisted of Gram-negative bacteria and a spirillum. It also utilised benzoate, the monohydroxybenzoates, protocatechuate and the cresols in similar conditions. (ring-¹⁴C) Phenol was converted into ¹⁴CO₂ and radioactive cell material; labelled *n*-caproate and acetate were also identified in the culture fluid. Bakker has, therefore, suggested that phenol is reduced to cyclohexanone followed by a hydrolytic scission of the alicyclic ring to *n*-caproate which can then undergo β -oxidation to give utilisable metabolites for cell growth, represented in Fig. 5. Methane was not produced in Bakker's cultures, presumably because of the presence of nitrate; Chmielowski and coworkers³³, however, have described a methanogenic fermentation of phenol.

A probable interpretation of events in the bacterial catabolism of aromatic compounds through anaerobic nitrate respiration may be: the reductive phase is accomplished by a ferredoxin-type reductant followed by the β -oxidation sequence and ring-cleavage to aliphatic acids; since these have to serve as carbon and energy source, a part must be oxidised and the resulting reduced coenzymes re-oxidised via a membrane bound proton-translocating redox system which is coupled by the electron transport chain to nitrate through nitrate reductase.

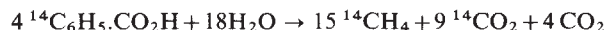
Methanogenic fermentation by a consortium

The formation of methane and CO₂ from aromatic natural products was the first observation to be made involving the biological destruction of benzenoid structures in strictly anaerobic conditions¹⁸. It can occur in the absence of nitrate, sulphate

and light through the action of an adapted microbial community. It is surmised that this phenomenon occurs widely in nature, in the processing of sewage and other waste effluents and in transformations of some pesticides.

Clark and Fina³⁴, Fina and Fiskin³⁵ and Roberts³⁶ working with benzoate as substrate and a consortium adapted from rumen-liquor and/or sewage-digester sludge, applied quantitative radiotracer techniques with the following results: (carboxy-¹⁴C) benzoate behaved like exogenous ¹⁴CO₂ in that it was not primarily reduced to methane. (Ring-¹⁴C,1)benzoate appeared mainly as ¹⁴CH₄; (ring-¹⁴C,4) benzoate was converted largely to ¹⁴CO₂. Propionate, acetate and formate were detected in the steam volatile fatty acid fraction of the fermentation liquor. The propionate was labelled in the carboxyl-C when (ring-¹⁴C,4) benzoate was used (L. R. Fina, personal communication), but not when (ring-¹⁴C,1) or (carboxy-¹⁴C) benzoates were substrates.

That the composition of the fermentation gases agrees with the stoichiometry demanded by the equation below was confirmed by Nottingham and Hungate³⁷ using (ring-¹⁴C) benzoate:



Although several workers had shown that benzoate-methanogenic cultures utilised certain members of the reductive pathway without a lag period, definitive chemical evidence for their participation was lacking until an abstract of the work of Keith³⁸ appeared. Isotopic trapping experiments enabled him to identify cyclohexanecarboxylate, cyclohex-1-enecarboxylate, heptanoate, valerate, butyrate, propionate and acetate in such cultures. He proposed that benzoate is reduced step-wise to cyclohexanecarboxylate followed by a reductive cleavage of the C₁-C₂ bond to give heptanoate; β -oxidation would then degrade this to valerate and propionate with the ultimate formation of acetate, one of the substrates for methane bacteria. Balba and Evans³⁹, using similar techniques, also detected the formation of cyclohexanecarboxylate, cyclohex-1-enecarboxylate, propionate and acetate from benzoate in both rumen and sewage-sludge methanogenic cultures; adipate was also identified in the latter fermentation liquor. Although there were about five other unidentified labelled acidic components present, none of these corresponded to heptanoate, *n*-caproate, valerate or butyrate. Prins (personal communication) and his collaborators have identified *trans* 2-hydroxycyclohexanecarboxylate, 2-oxocyclohexanecarboxylate, pimelate, caproate, butyrate, acetate and molecular hydrogen as metabolites of benzoate in methanogenic cultures adapted from the black mud of a polluted river.

Whether the methanogenic fermentation of benzoate is accomplished entirely by species of methane bacteria or if it requires the cooperation of other members of the consortium, remained an open question until recently. In an elegant study, Ferry and Wolfe⁴⁰ observed that *o*-chlorobenzoate inhibited benzoate degradation without affecting the production of methane from acetate; the uncoupling of these two steps argues in favour of a microbial food chain. Quantitative studies on the rate of ap-

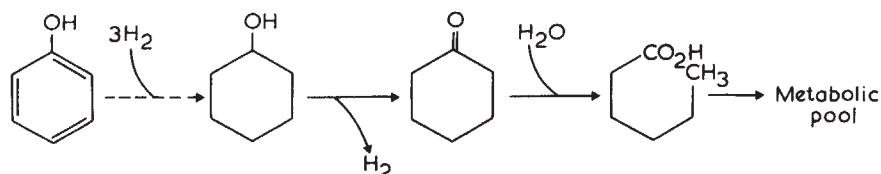
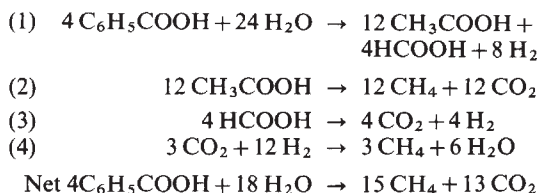


Fig. 6 Anaerobic metabolism of phenol through *n*-caproate by a consortium in the presence of nitrate. Initial reduction accomplished by an unidentified hydrogen donor (redox potential -0.42 V).

pearance and disappearance of intermediates and formation of products using ^{14}C labelling where necessary, enabled these workers to propose the following steps in the overall conversion of benzoate to methane:



A careful study of their consortium which had been subcultured for a few years revealed three predominant methanogenic organisms. *Methanobacterium formicum* and *Methanospirillum hungatei* were obtained from the floc in pure culture, but the acetate-degrading methanogenic bacterium defied attempts at isolation. None of these utilised benzoate. A facultative Gram-negative organism which utilised benzoate aerobically was also present, but it failed to grow on this substrate anaerobically even in the presence of nitrate. Their cultures produced methane without a lag-period from cyclohexanecarboxylate, 2-hydroxy-cyclohexanecarboxylate, salicylate, pimelate and acetate; cyclohex-1-enecarboxylate did not behave this way. Ferry and Wolfe⁴¹ point out that the energetics of benzoate degradation make it obligatory for the methane bacteria to utilise the acetate produced if the facultative benzoate-degrader is to obtain energy for growth in conditions which exist in the culture.

In the methanogenic cultures of Balba and Evans³⁹, electron microscopy revealed methanogenic bacteria similar in morphology to those already described by Ferry and Wolfe⁴⁰. The facultative Gram-negative organism present in our consortium grew aerobically on *p*-hydroxybenzoate but not on benzoate. It was, nevertheless, able to degrade benzoate anaerobically in the presence of nitrate (compare Williams and Evans³¹). Our cultures continued to produce a gas mixture of approximate composition, CH_4 (54%), CO_2 (46%) without a lag-period when benzoate was replaced by alicyclic intermediates of the reductive pathway, the hydroxybenzoates, phloroglucinol, cinnamate, phenylalanine and even shikimate or quinate.

It has now been established that in all these mixed cultures which ferment aromatic compounds to methane and CO_2 , the benzene nucleus is first reduced and then cleaved to aliphatic acids by facultative Gram-negative organisms; these acids are then

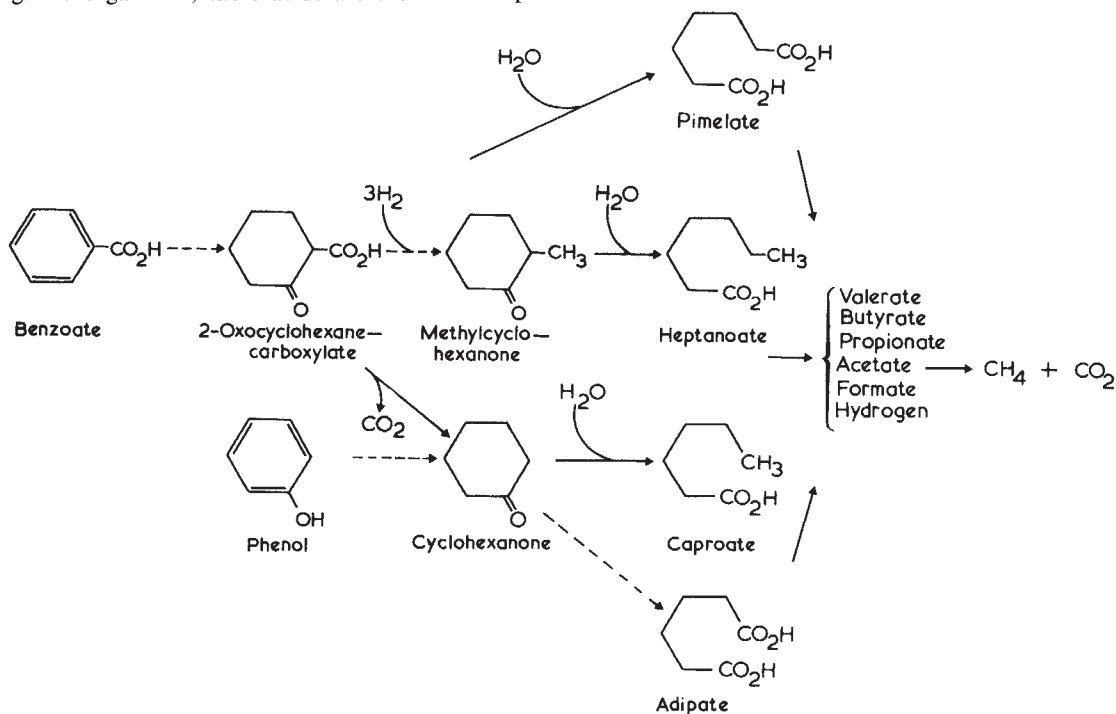
converted to suitable substrates for various methane bacteria to complete the process. The electrons generated are probably excreted as H_2 gas which is used for the reduction of CO_2 to CH_4 . Pure cultures of all the necessary bacteria from these consortia are not yet available for the deliberate reconstitution of the community. It is unlikely that the consortia studied by different workers are absolutely identical in bacterial species composition, yet almost all investigators find the same alicyclic intermediates. The fatty acid anomaly, the occurrence of heptanoate in Keith's benzoate-methanogenic cultures could have other explanations for its origin rather than the one postulated, the reductive cleavage of cyclohexanecarboxylate, a reaction for which there is no biochemical precedent, Fig. 7 shows these intermediates.

Inocula from anaerobic environments where methane gas formation occurs, can be adapted to the methanogenic fermentation of benzoate in about 1–2 months; an initial addition of acetate as well to the culture shortens this period considerably. This does not mean that the benzene nucleus is actually degraded in all these anaerobic environments, for example, benzoate and the aromatic amino acids do not give rise to methane in the rumen; they are excreted as urinary hippurate and other conjugates. Shikimate and quinate are, however, reduced by rumen microflora to cyclohexanecarboxylate which appears in the urine of herbivores as hexahydrohippurate⁴¹.

The rate-limiting steps in the biological degradation of natural aromatic polymers occur at the stage when it is necessary to dismember the lignins and tannins into their small molecule aromatic components. (Artificial treatment of ligno-cellulosic material with alkali at an elevated temperature results in a much improved utilisation of their phenolic components by methanogenic fermentation⁴².) Extracellular microbial enzymes slowly achieve this aerobically, although there is a paucity of precise biochemical information about these reactions for obvious reasons^{43,44}. The catechin tannins present an additional obstacle, because of their tanning properties.

It is difficult, at present, to assess the relative importance of the aerobic and anaerobic methods of decomposition of aromatic compounds in the biosphere. Regarding the latter, the *Athiorhodaceae* require light and anaerobiosis for it to be possible; such habitats exist in oceans and lakes⁴⁵. Soils afford anaerobic conditions frequently generated by impeded drainage or flooding (natural or deliberate); soil aggregates (crumb structure) are anaerobic internally—where the appropriate bacteria reduce NO_3^- and SO_4^{2-} . Whether SO_4^{2-} can also act as a terminal electron acceptor for anaerobic catabolism of aromatic substrates has not

Fig. 7 Probable pathways in the fermentation of benzoate and phenol by adapted bacterial consortia from a variety of methanogenic ecosystems. 1-Methylcyclohexanone is a hypothetical intermediate, but is one of a few possible precursors of heptanoate; others involve reduction of C.2 after a cleavage, or a biosynthetic origin.



yet been convincingly demonstrated⁴⁶; attempts in the authors' laboratory using *Desulphovibrio* were unsuccessful. Microbial communities in habitats where methane formation occurs abound in nature; they can also be made to produce the gas from a variety of aromatic substrates in the laboratory. Dutton and Evans⁴⁷ found that appreciable concentrations of fatty acids inhibited the photometabolism of benzoate—a temporary phenomenon, attributed at the time to competition for coenzyme A. In natural anaerobic environments, cellulose fermentation takes precedence over the degradation of aromatics.

Many factors are concerned in the degradation of organic compounds, irrespective of whether they are of natural or synthetic origin. They are all exposed to physico-chemical forces and biological agents capable of causing chemical change in the biosphere⁴⁸. Dilution processes and time eventually ensure that conditions are created for these factors to operate. Concentrations of noxious chemicals may, however, accidentally reach levels in local areas which are extremely hazardous to life; these require exceptional decontamination measures and legislation. Although many useful pesticides are developed empirically, their mode of action usually depends on a selective interference with some fundamental cellular process. Their structures, apart from certain uncommon substituents (for example, halogen) also frequently bear some resemblance to key biochemicals concerned in metabolism. Some linkages may, therefore, be amenable to attack by constitutive group-specific microbial enzymes. It is a common observation that many of these foreign chemicals are degraded more rapidly by a mixed adapted community of soil microorganisms than by any of the freshly isolated individual members of the group—for example many chlorinated hydrocarbon pesticides under suitable biologically-active anaerobic conditions^{49,50,51}; Dalapon⁵² (2, 2-Dichloropropionate) and Asulam (M. Khan and W.C.E., unpublished) [Methyl-(4-aminobenzenesulphonyl)carbamate] under aerobic conditions.

A microbial community reacts to the presence of xenobiotic organic chemicals in subtle ways, depending on their structures and the environment; several biological devices are brought into play which ultimately cause their disappearance. Among the known stratagems used are: co-metabolism⁵³, enzyme induction⁵⁴, transfer of metabolic plasmids^{55,56}, mutation leading to the evolution of enzymes with new specificities and activities^{57,58} and microbial interactions⁵⁹ as yet imperfectly understood but operating in the methanogenic consortia which anaerobically degrade aromatic substrates.

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articles

Multichannel seismic reflection profiles of the continental crust beneath the Newfoundland Ridge

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Multichannel seismic reflection profiles extending south from the Grand Banks indicate that large 'basement' features underlying the Newfoundland Ridge are composed of sedimentary strata. The Newfoundland Ridge, therefore, is interpreted as an area of subsided continental crust rather than a ridge of oceanic crust. The J magnetic anomaly intersects this complex, and the physiographic ridge associated with this anomaly may also be continental in origin.

HYPOTHETICAL reconstructions of the North Atlantic before Mesozoic-Cainozoic seafloor spreading define the south-western edge of the Grand Banks structural block as a transform margin that developed when the African plate was displaced from that region^{1,2}. The Newfoundland Ridge (Fig. 1) has been interpreted as the trace of a fracture zone in oceanic crust marking the continuation of this transform margin^{3,4}. The Newfoundland Ridge is a physiographic feature that extends about 900 km south-east from the southern tip of the Grand Banks, with a subsidiary projection, the 'Spur' ridge or 'J-anomaly' ridge, extending to the

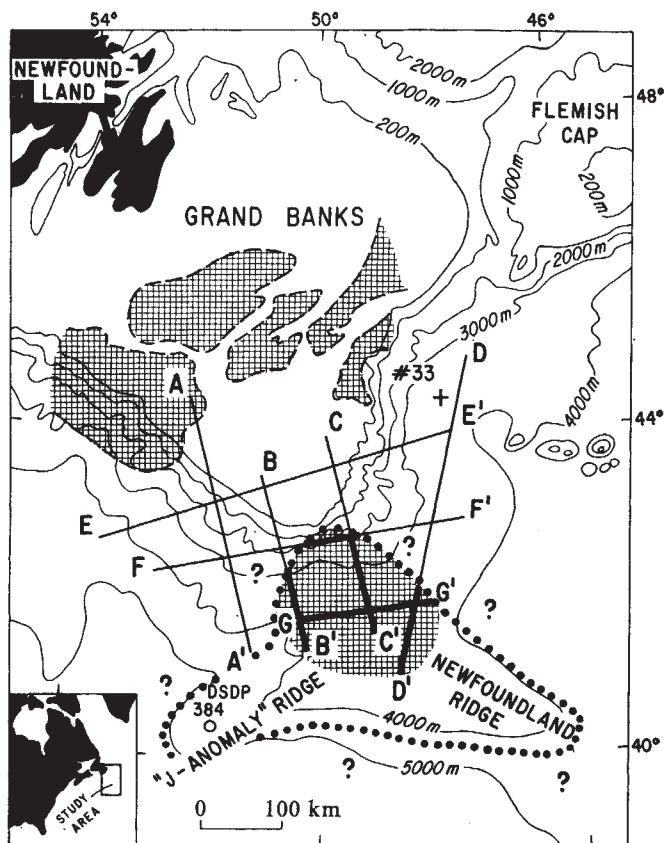


Fig. 1 Bathymetric map of the Grand Banks region (contours in metres). Lettered lines show the locations of the profiles drawn in Fig. 2. The heavy parts of these lines and surrounding crosshatch denote areas where seismic reflectors are apparent beneath event U. The dotted line indicates the minimum extent of sedimentary strata beneath the Newfoundland Ridge inferred on the basis of physiography. Question marks denote the possibility of founded sialic crust adjacent. Cross-hatched areas enclosed by dashed lines indicate the extent of sub-unconformity basins of the Grand Banks. The circle and cross indicate DSDP Site 384 (ref. 11) and sonobuoy station No. 33 (ref. 10).

south-west. Watson and Johnson⁵ interpreted seismic profiles from the Newfoundland Ridge as showing buried basement blocks, which had been uplifted and distorted by faulting. From the magnetic anomalies associated with these blocks they inferred a volcanic origin. Ballard *et al.*⁶ have described topographic and structural features characteristically associated with the high amplitude (500–1,000 nT) J-magnetic anomaly. Hall⁷ and Keen *et al.*⁸ have suggested that the J-magnetic anomaly continues north-eastwards with slight offset, across the Newfoundland Ridge. These investigations generally imply an oceanic origin for the Newfoundland Ridge. This paper presents an interpretation of multichannel (24) common depth point reflection seismic data that supports an alternative, continental origin for this feature. These seismic data (Fig. 1) are part of a nonproprietary survey by Seiscan Delta Ltd that the Canadian Government has purchased for the purpose of regional control. Figure 2 shows reduced tracings of reflectors on the processed record sections. The vertical scale magnification of the bottom profile in these diagrams is about 6 to 1. Magnetic data (also from Seiscan Delta) are plotted as anomaly profiles with several of the seismic sections. Figure 3 is a reproduction of a portion of a seismic record section on the Newfoundland Ridge.

Interpretation of data

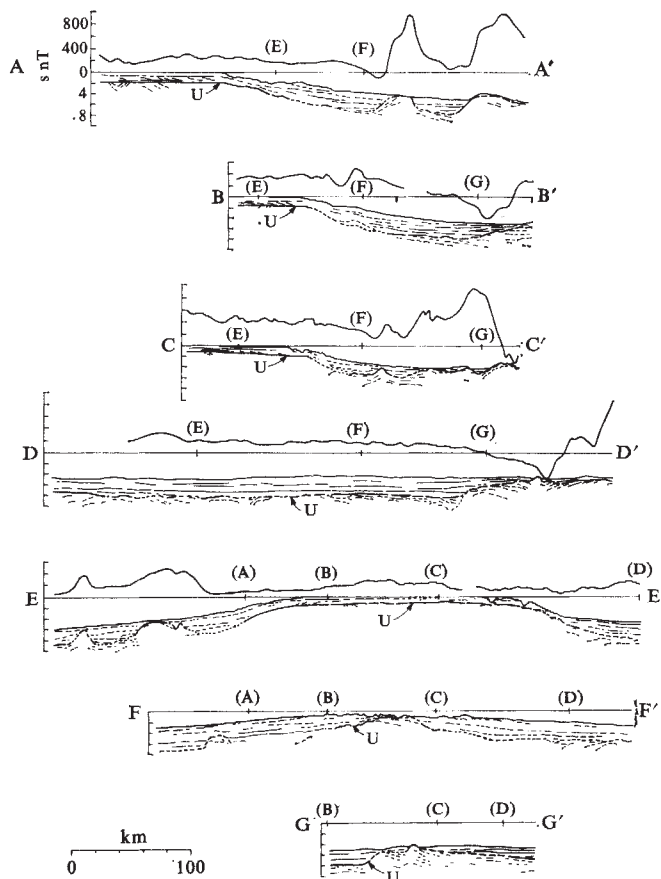
The subsurface geology of the Grand Banks has been outlined through geophysical surveys and exploratory drilling for petroleum^{1,9}. A principal element of Grand Banks geology is a late

early Cretaceous unconformity, which separates a blanket of Cretaceous and Tertiary sediments from older rocks in sub-unconformity basins and intervening areas of basement (Fig. 1). This unconformity is an excellent seismic boundary. On the profiles in Fig. 2 the seismic reflections from the unconformity—(event U) have been traced from the shelf to deep water. On individual seismic records it is usually difficult to follow this event through the zone of the shelf break, where the change in bottom slope and probable changes in sediment lithology combine to degrade record quality. Fortunately, however, the correlation of the U event from the shelf to deep water can be checked by crossreferencing the intersecting lines of survey coverage (Fig. 1).

Section G and the southern ends of sections B to D (Fig. 2), located on the Newfoundland Ridge, show seismic reflectors to depths approaching 4 s (two-way time) below the U event, with no indication of any underlying basement. These sub-U reflectors are interpreted as denoting gently dipping sedimentary strata. Velocity analyses performed in the course of data processing indicate velocities in these strata as low as 3–4 km s⁻¹, which would yield a minimum thickness for these strata of 6–8 km. The apparent attitudes of the seismic reflectors at the crossing of lines C and D with line G indicate a north-westerly strike, approximately parallel to the physiographic trend of the Newfoundland Ridge. These strata form cuestas and hogbacks at the U event interface; however, it is not clear from the seismic data whether the scarps associated with these features are entirely erosional or whether displacement by faulting has occurred. Dipping reflectors below the U event in the central part of line F, between crossings with lines B and C, are also interpreted as originating in sedimentary strata.

Sub-U penetration of a different character occurs on the eastern end of line E, and along the northern three-quarters of line D (Fig.

Fig. 2 Seismic and magnetic profiles from locations indicated in Fig. 1. Vertical scale for the seismic profiles is in seconds of two-way travel time. The U event has been emphasised. Vertical bars on profile B indicate coverage of Fig. 3.



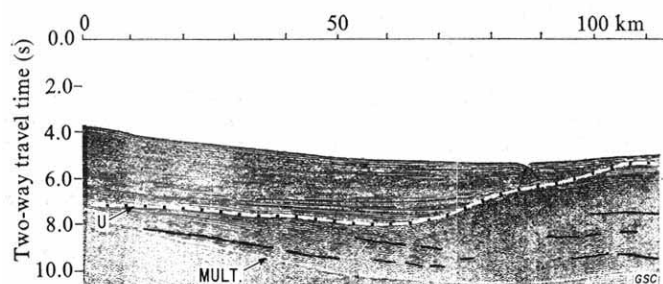


Fig. 3 Photograph of a portion of a seismic record section on the Newfoundland Ridge.

2). These reflectors show variable attitudes, amplitudes and continuity. Beneath the south-western slope of the Grand Banks, large anomalies traced by the U event on lines A and E have been interpreted by Jackson *et al.*¹⁰ as relief on oceanic basement. The seismic record sections show reflectors on the flanks of these features, but no apparent penetration beneath their central, highest parts. The character and disposition of these flanking reflectors give the impression of talus deposits.

The reflection seismic data described above distinguish between acoustic basement highs beneath the south-western slope of the Grand Banks and blocks of sedimentary strata beneath the Newfoundland Ridge. Available magnetic coverage⁷ shows a complex assemblage of large magnetic anomalies associated with both areas, and with the J-anomaly ridge as well, but does not seem adequate to refine the limits of these two types of sub-U structure indicated by seismic data. Possibly there is a north-westerly trend to the magnetic anomalies in the vicinity of the Newfoundland Ridge.

Occasional penetration of seismic energy is seen beneath the U event on the Grand Banks (sections A, B, C and E, Fig. 2). The north end of section A shows more extensive penetration associated with the South Whale sub-basin (Fig. 1). Excellent seismic records across the sub-unconformity basins on the Grand Banks have been presented by Amoco and Imperial⁹.

Discussion

Assuming that the U seismic event has been traced correctly from the Grand Banks to the limit of coverage in deep water, and that the chronological significance of this event remains approximately constant, then the age of the sedimentary strata underlying the U event on the Newfoundland Ridge must be early Cretaceous or older. The U event unconformity is evidence that these strata were exposed to subaerial weathering before the subsidence time. The results from DSDP site 384, on the J-anomaly ridge (Fig. 1), indicate that subsidence of at least 4 km has affected this area since the late early Cretaceous¹¹. The apparent thickness of the strata beneath the U event on the Newfoundland Ridge (up to 6–8 km) is comparable to the thickness of section that occurs in the sub-unconformity basins on the Grand Banks, and the areal extent of these strata as defined by the seismic lines in Fig. 2 is at least equivalent to that of the smaller basins on the Grand Banks (Fig. 1). It is, therefore, postulated that the sedimentary strata underlying the U event on the Newfoundland Ridge may represent a basal accumulation analogous to the sub-unconformity basins on the Grand Banks, which has subsided relative to the Grand Banks by displacement across a hinge line or zone of faulting beneath the present continental slope. If these strata extend to the physiographic limit of the Newfoundland Ridge (Fig. 1), this feature would represent a subsided, remnant basin roughly equivalent in area to the total of the several remnant basins on the Grand Banks.

The sub-unconformity basins on the Grand Banks are fault-bounded grabens and half-grabens containing Jurassic and older formations, with structural complications related to salt diapirism⁹. The sparse data available indicate that the basement rocks underlying these formations are composed of a wide variety of lithologies and probably range in age from Palaeozoic to

Precambrian^{1,9}. These data are a meagre basis for speculation as to the nature of the basement rocks that underlie the sub-U sedimentary strata on the Newfoundland Ridge, except that they are probably sialic. But, an important implication of the inferred basal setting of these strata is that the basement rocks adjacent to the basin subsided more deeply than the sediments, and that foundered sialic rocks thus extend some distance laterally from the Newfoundland Ridge.

The multichannel seismic coverage (Fig. 1) is not sufficient to determine the extent of sialic crust laterally from the Newfoundland Ridge, but variations in the character of these seismic data in the area peripheral to the ridge may be relevant. Off the eastern margin of the Grand Banks the northern three-quarters of line D (Fig. 2) shows irregular and discontinuous reflectors beneath event U. The depth to the U event opposite sonobuoy Station No. 33 (Fig. 1) of Jackson *et al.*¹⁰ corresponds closely to the depth to their inferred oceanic basement refractor of 5.82 km s⁻¹. On the south-western side of the Grand Banks Jackson *et al.*¹⁰ recorded a mantle refraction at a depth of approximately 17 km, and described the irregular surface traced by the U event in that area (lines A and E, Fig. 2) as oceanic basement. The refraction seismic definition of oceanic basement in these two areas obviously is not sensitive to the contrasts in surface profile and seismic penetration shown by the multichannel reflection seismic records, and it is doubtful that both of these refraction measurements define true oceanic basement. Possibly the differences in the reflection seismic character of these two areas relate to variation in the composition of parent sialic crust, now subsided.

The extent of subsided continental crust peripheral to the Newfoundland Ridge can be examined in a larger context. Pre-drift fits of the North Atlantic tend to place the western margin of the Iberian Peninsula opposite the south-western margin of the Grand Banks^{1,2}. In some reconstructions, Galicia Bank, which lies offshore west of the northern part of the Iberian Peninsula, obstructs the tight juxtaposition of these two margins and leaves an untidy gap to the south. An area of continental crust about the Newfoundland Ridge would help to fill this gap.

The large magnetic anomalies in the vicinity of the Newfoundland Ridge may relate to the presence of subsided continental crust as hypothesised above. Possibly these large anomalies express the susceptibility contrast between sedimentary rocks and volcanic rocks, assuming that the sediments and sialic basement rocks were invaded by mafic volcanics, probably at the time of subsidence. This interpretation may also apply to the physiographic and magnetic characteristics of the J-anomaly ridge, which coincides with a larger magnetic anomaly than expected in relation to its physiographic relief and reasonable magnetisation values for oceanic basement¹¹. The distinctive topographic aspects of the J-anomaly ridge⁶, particularly the anomalously flat surfaces apparently disrupted by faults, would be normal features for a ridge composed primarily of sedimentary strata. This postulated origin for the J-anomaly ridge implies that it does not necessarily have an isochronal relationship to seafloor spreading. Hall⁷ and Keen *et al.*⁸ have discussed the problematic nature of the age of the J anomaly, which has been assigned ages ranging from 115 to 137 Myr BP.

Conclusions

Multichannel reflection seismic data from the Newfoundland Ridge allow penetration below the surface commonly regarded as oceanic basement, with important consequences for the geological interpretation of this region. These data indicate that the Newfoundland Ridge is composed of blocks of sedimentary strata, pre-late early Cretaceous in age, which may represent a sedimentary basin, now subsided, analogous to the sub-unconformity basins on the Grand Banks.

A synthesis of available data from the region of the southern Grand Banks suggests that sialic crust adjacent to the Newfoundland Ridge has subsided to oceanic depths, and that variations in its composition are shown by variation in reflection seismic character. These differences are beyond the resolution capability of the refraction seismic method. If it is accepted that the

subsidence of sialic crust was accompanied by igneous invasion, a new pattern of magnetic anomalies probably was generated, with their orientation determined by the mechanical processes of subsidence and the geological fabric of the parent crustal material. These anomalies would not be expected to have chronological significance in terms of processes of seafloor spreading.

Additional survey control is required to map the limits of postulated subsided continental crust in the vicinity of the Newfoundland Ridge. Probably it will be necessary to apply common depth point reflection seismic techniques to effectively pursue this problem. Increased application of the common depth point reflection seismic method in the deep sea, particularly in the marginal zones of the ocean basins, will reveal further variations in the character of oceanic basement. The data described here indicate another probable sliver, chip or mini-plate that geomet-

ricians must accommodate in closing the North Atlantic to predrift configurations.

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Absolute radiocarbon dating using a low altitude European tree-ring calibration

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Attempts to produce a calibration curve for the radiocarbon timescale by analysis of known age materials have highlighted the inaccuracies of conventional radiocarbon dating methods. The resulting ambiguities have caused a loss of confidence in radiocarbon dating particularly among European prehistorians. We describe here an absolute radiometric dating technique involving the investigation of all probable sources of error. Accurate measurements were made with an overall precision of less than 25 yr standard deviation, on a floating North of Ireland tree-ring chronology.

CORRECTION for natural atmospheric radiocarbon variation is only one of the several corrections that have been necessary to relate the radiocarbon assay of organic materials to a calendar time scale. The assumption of the constancy of natural radiocarbon was first questioned by DeVries¹, and several teams set out to test the constancy by measuring the radiocarbon content of known age samples. Willis, Tauber and Munnich² and Suess³ showed that there was a significant trend away from the theoretical relationship. The bristlecone pine chronology was used for further investigation of the problem^{4,5}. Suess showed major short-term variations or 'wiggles' in the calibration and suggested that in certain periods radiocarbon measurements could not provide unambiguous dates.

Further bristlecone pine samples were then distributed to three laboratories; La Jolla, Arizona and Pennsylvania and the results presented in 1969⁶. Suess⁷ presented his calibration as a curve drawn by eye through the measurement points, incorporating a number of pronounced wiggles. Part of this graph is reproduced in Fig. 1. Following publication several authors⁸⁻¹¹ questioned the validity of Suess's line.

During the next few years few new data were produced from the radiocarbon laboratories and discussions continued among radiocarbon users on the validity of the wiggles and on what methods should be used to apply corrections to radiocarbon age measurements^{12,13}. At various times the bristlecone pine tree-ring chronology was questioned, but this was resolved by the replication of the greater part by LaMarche and Harlan¹⁴. The possibility was also raised that *in situ* production of ¹⁴C in the high altitude bristlecone pines was leading to spurious results. This has been discounted both by experiment¹⁵ and by theoretical calculations based on the nitrogen content of the wood¹⁶. Two more recent calibrations are given by Damon *et al.*¹⁷ who present a

calibration based on 549 samples using a curvilinear regression, and by Clark¹³. The latter summarises previous calibrations and examines the evidence from duplicate measurements which indicate a greater spread in the results than can be accounted for by the stated precisions. Disagreements between radiocarbon dates for bristlecone pine wood and for same-age, historically dated, Egyptian material are apparent. These disagreements led McKerrell¹⁸ to suggest that systematic differences may exist and that the bristlecone calibration may not be appropriate in European contexts.

Although it is 19 years since the DeVries report, there is still no satisfactory experimentally-derived calibration that can be used unambiguously to convert radiocarbon ages to calendar dates. The publication of numerous calibration curves based on identical material, has proved that laboratories can only agree on the general trend of the calibration. Because of these disagreements it has been left to statisticians to provide smoothed calibrations. One such attempt has been the fit by Damon¹⁷ of a third order orthogonal polynomial to experimentally derived data. At a recent conference no internationally acceptable radiocarbon calibration could be arrived at. It was apparent that an attempt should be made to resolve these discrepancies on the assumption that the experimental method was at fault.

By 1970 studies on oaks had established that long tree-ring chronologies could be constructed in the North of Ireland. To exploit this source of low altitude calibration material, a radiocarbon dating system capable of taking advantage of large sample size was designed; the method chosen was the liquid scintillation counting of benzene synthesised from sample cellulose. In 1972 a research programme was started to investigate all factors likely to contribute to the inaccuracy of radiocarbon measurement. Details of the procedure for synthesising large volume benzene samples and methods of counting will be given elsewhere (G.W.P., in preparation). So that the results given here may be critically examined, the corrections derived from these investigations are given in Table 1.

Methods

The measurements were carried out on a 1,200-yr section of a 2,990-yr floating tree-ring chronology derived from sub-fossil oaks which grew below 130 m altitude between latitude 54° and 55°N and longitude 6° and 8°W (ref. 19). The chronology is based on some 300 trees and is replicated at all points. Because of bulk-field sampling and relatively large annual rings it was possible to produce 20-yr units of 180-200 g for radiocarbon measurement. This is in comparison with normal bristlecone pine samples of 20 g.

Table 1 Experimentally derived corrections for significant error sources

Error source investigated	Correction derived experimentally	Normalised value	Estimated error on correction
Barometric pressure variation	Background increases by 0.0127 c.p.m. per mbar decrease in* pressure	1010 mbar	± 0.013 c.p.m.
Background variations with weight loss	Background decreases by 0.0003 c.p.m. per mg decrease in weight	13.1325 g C ₆ H ₆ 14.2666 g C ₆ H ₆ + scintillant	± 0.003 c.p.m.
Background variations with purity	Changes by 0.041 c.p.m. per cent change in channels ratio	External source channels ratio of 1.95	± 0.041 c.p.m.
Efficiency variation with weight loss	Efficiency increases by 0.00104% per mg decreases in weight	13.1325 g C ₆ H ₆ 14.2666 g C ₆ H ₆ + scintillant	$\pm 0.010\%$ of nett c.p.m.
Differential loss of sample from mixture and correction to normalised weight including all weighing errors†	98.3% of loss from vial is sample benzene	13.1325 g C ₆ H ₆	$\pm 0.076\%$ of nett c.p.m.
Efficiency variation with purity	‡%Deviation from normal purity 0.0–1.0 (1.00005) 1.0–2.0 (1.00015) 2.0–3.0 (1.00025)	External source channel ratio of 1.95	$\pm 0.005\%$ of nett c.p.m.
Mass spectrometry correction	Isotopic enrichment measured (actual reproducibility over 12 months)	–25% rel. P.D.B. (samples) –19% rel. P.D.B. (oxalic reference)	$\pm 0.05\%$ of nett c.p.m. $\pm 0.05\%$ of nett c.p.m.
Individual vial difference for efficiency	Using high ¹⁴ C labelled C ₆ H ₆	Mean value	$\pm 0.029\%$ of nett c.p.m.
Individual vial difference for background	95% limits of parent distribution	Mean value	± 0.058 c.p.m.

The method used for calculation was that given by Callow *et al.*²⁷.

*High correlation of background with barometric pressure was found. Failure to correct could lead to a bias of up to 100 yr and make use of long-term mean background values impossible.

†Large inaccuracies, up to 40 mg, can be caused by the effect of humidity and temperature on plastic caps.

‡Correction factor given in parentheses.

Measurements were made on contiguous 20-yr increments taken from different trees in the floating chronology. Each increment was reduced to shavings and treated with a chlorite bleach²⁰ which left a sample free from tannins and lignins and close to pure cellulose. This treatment has been shown¹⁶ to remove the greater part of any effect of heartwood deposition in oak since cellulose is not laid down at the heartwood/sapwood transition. Following cellulose preparation samples were charred at 450 °C to leave a carbon residue and converted to CO₂ by combustion or bomb calorimetry. The gas was sub-sampled for mass spectrometric measurement of the stable carbon isotope ratio.

The synthesis of benzene from sample CO₂ followed the conversion path CO₂ → LiC → C₂H₂ → C₆H₆ using the method of Barker²¹. This method was modified to allow the synthesis of up to 20 ml of sample benzene. Contamination of the water supply, and of the lithium metal used in the synthesis with ²²²Rn was severe and great care was needed to ensure its removal. Mass spectrometric analysis of the synthesised benzene showed that it was at least as pure as scintillation grade benzene and was of consistent quality.

The samples were measured on a Philips 4510 automatic liquid scintillation analyser with the three channels for individual isotope monitoring set for the measurement of ³H, ¹⁴C and ²²²Rn. Two extra channels were used for the differential energy measurement of a 7 μCi ¹³³Ba external source, the ratio being used to check variation in pulse height (a limit of $\pm 4.5\%$ of mean purity pulse height was applied). Balance point counting of ¹⁴C (a differential technique in which the pulse height spectrum within the selected channel is so balanced that small variations in pulse height contribute almost as many pulses to the channel as are lost) reduced the effect of pulse height variation giving long-term changes in efficiency of less than 0.03%. Continuous background

measurement of a flame-sealed vial showed no significant deviation from a normal distribution when corrected for barometric pressure variation (a major source of error, see Table 1). Gain stability checks were maintained using flame-sealed vials containing high specific activity ³H and ¹⁴C-labelled toluene. Because of the relative increase in pulse height of toluene to benzene the ¹⁴C high standard was not measured in a balance point position and was therefore very sensitive to gain changes. The ¹⁴C channel was set so that balance point was achieved at a specific purity equivalent to an external source channels ratio of 1.95. Each sample was normalised to this purity (Table 1).

The vials used were of standard low potassium glass selected for weight similarity from a batch of 500. The background for each vial was monitored for 2-yr and was checked after each sample or standard filling. The vials were undercoated with white paint above the 18 ml level to improve photon collection and to give a precise counting geometry and then coated with black paint to reduce optical feedback between one photomultiplier and the other. Each vial was filled to within ± 5 mg of the normalised weight for benzene and scintillant (Table 1). The scintillation mixture in 1 ml of toluene added to 15 ml of sample benzene (13.1325 g) gave a scintillant concentration of 10 g l⁻¹ butyl PBD and 0.6 g l⁻¹ PBBO. An 'Agl' pipette with hypodermic needle was used to inject the sample through a special filling cap which was then replaced with a sealing cap incorporating discs of rubber, PTFE and pure tin. All samples were calculated using the Libby half life and 95% of the oxalic acid reference standard count rate representing AD 1950.

Each vial background was measured for successive 100-min intervals giving an accumulated count of approximately 100,000 for each vial. Each 100-min count was corrected for barometric

pressure variation and plotted to give a quality control distribution for that vial. Mean values of the vial distributions were compared in a single 'parent' distribution having a standard deviation equal to $\pm 0.3\%$. The points fell into an apparent normal distribution indicating that all the vials belonged to the same distribution thus allowing a single grand mean background value to be used. The values of the background and its precision including all errors and corrections were: 9.289 ± 0.078 c.p.m.

Standards, samples and backgrounds were counted in sequence. Two oxalic reference standards were measured at any one time providing continuity and overlap during their regular replacement. Each measurement was corrected and plotted on a quality control chart, and a mean value determined for each individual oxalic distribution. As a check for unique vial efficiencies, high specific activity samples were used. On an observed count of not less than 1.6×10^7 c.p.m. for each vial, no significant difference was obtained. The mean oxalic distributions were then compared and were found to lie within ± 2 standard deviations of a grand mean value, once more signifying that they probably belonged to a single distribution. The mean value of this distribution for the nett reference standard count rate used in the date calculation was: 122.854 ± 0.163 c.p.m.

After synthesis each sample was left to stand at -10°C for at least 1 month before being prepared for counting, to allow a 100-fold decrease in any remaining ^{222}Rn contamination. At least 300,000 sample counts were accumulated over a minimum counting period of a fortnight. As in the case of standards and backgrounds, the barometric pressure and pulse-height variation were measured during each cycle and used for the correction of each result. Correction for weight changes was made after vial removal; any changes in cap weight being assessed and corrected for. All measurements were normalised to the value quoted in Table 1. The calculated dates were finally corrected for isotopic fractionation.

The 25-yr standard deviation quoted on all dates is a blanket precision covering all errors found to be significant in the experimental method. It includes not only the counting statistics and mass spectrometric correction normally quoted (equivalent in this case to less than ± 17 yr), but also the estimated errors due to all the corrections listed in Table 1. Some of the sources of error, if not corrected for, could cause a bias of up to 100 yr.

Results and discussion

The results of the ^{14}C measurements are given in Table 2, in yr before AD 1950. Some results for the contiguous series of samples are not yet available due to random selection of samples for

Table 2 Radiocarbon dates b.p. for 20-yr increments of tree-ring dated Irish oak wood

Tree-ring age (yr)	^{14}C date (b.p.)	Tree-ring age (yr)	^{14}C date (b.p.)	Tree-ring age (yr)	^{14}C date (b.p.)
0	3,604	400	3,969	800	4,240
20	3,600	420	4,007; 4,028	820	4,304
40	3,622	440	—	840	—
60	3,731; 3,722	460	3,981	860	4,376
80	3,669	480	4,017	880	4,399
100	3,728	500	4,059	900	4,390
120	3,714	520	4,081	920	4,347
140	3,737	540	4,107	940	4,387
160	—	560	4,072	960	4,466
180	3,792	580	4,161	980	4,438
200	3,801	600	4,125	1000	—
220	3,832	620	4,205	1020	—
240	3,856	640	4,115	1040	—
260	3,859	660	4,133	1060	4,499
280	3,875	680	4,146	1080	—
300	3,878	700	4,225	1100	4,517
320	3,906	720	4,179	1120	4,541
340	3,861; 3,884	740	—	1140	4,495
360	3,883	760	—	—	—
380	3,977	780	4,203	—	—

Tree-ring time scale is floating. 0 on this scale = 5,810 on the computer scale given in Pilcher *et al.*¹⁹. ^{14}C dates are calculated on the 5,568 yr half life. 1 σ precision on all dates is ± 25 yr.

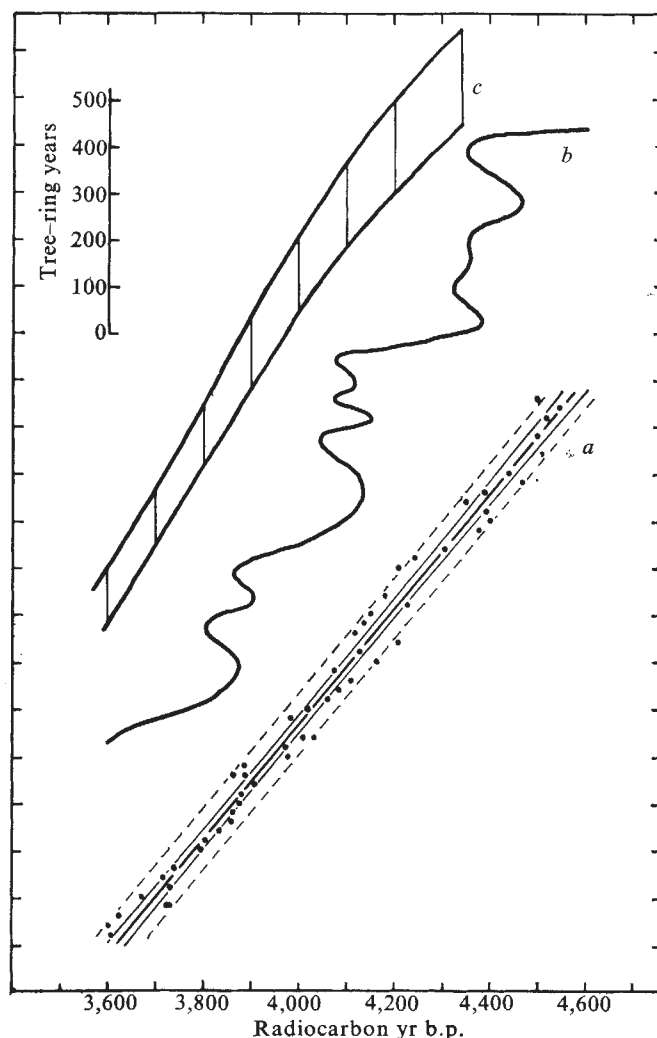


Fig. 1 Relationship between radiocarbon and tree-ring years. *a*, Relationship established using low-altitude oak from the North of Ireland; two standard deviation limits dashed, regression line drawn with curvilinear 95% confidence limits. *b*, Curve published by Suess⁷ based on bristlecone pine wood. *c*, Historically derived calibration based on Egyptian material published by McKerrell¹⁸.

measurement, but no results have been deleted or ignored. The measurements are plotted on Fig. 1 together with the regression line through them. The regression line has a slope of 1.215, and a regression coefficient of 0.99. The *F* value for the regression is greater than 3,000 indicating a good straight line fit to the distribution. Also shown on Fig. 1 are sections of some previous calibration curves. Each is drawn on the same radiocarbon axis and on its own independent tree-ring axis. This is necessary because our tree-ring sequence is not yet tied to the present day.

The investigations carried out in preparation for these calibration measurements showed significant error sources in conventional ^{14}C measurement techniques (G.W.P., unpublished). The results given here show that these error sources can be overcome and that the overall one standard deviation measurement precision can be reduced to less than 25 yr. The closeness of fit to a straight line indicates that there are no remaining significant random error sources not covered by the measurement precision. By increasing the sample counting time the precision could be reduced to the same level as other error sources giving an overall one standard deviation limit of ± 12 yr in 10,000, and similarly with a long count the limit of detection would be greater than 60,000 yr. If, as these results suggest, each radiocarbon age represents a unique calendrical age at least over a large part of the ^{14}C time scale, then a very close approximation can be made to an absolute time scale.

The straight-line slope through our results lies within 1% of that derived by Damon *et al.*¹⁷ and is close to the lines deduced by

Wendland and Donley⁸ and by Clark¹³ on the basis of the bristlecone pine results. The line based on Irish material does not show, within measurement limits, any of the wiggles predicted by Suess⁷ for this period or retained by McKerrell¹⁸ in his 50-yr averaging of the bristlecone pine data. This rules out, over the period investigated, the use of wiggle matching²² as a dating technique. Suess (personal communication) has criticised our use of 20-yr blocks of tree-rings as he maintains that this will cause some smoothing of the wiggles. By using contiguous 20-yr blocks, however, no information is lost and the only way that short-term variations could produce a straight line calibration would be if they occurred as an exact harmonic of a 20-yr cycle over the whole 1,200 yr. This is inherently unlikely. Also, most radiocarbon samples represent greater than this time-span. Archaeological samples, which are very often of oak in European contexts, are normally charred branches or small timbers of at least 20 yr. Peat and sediment samples used for palaeoecological studies are normally of greater than 20-yr growth. The effect of smoothing due to deposition of recent carbon at the heartwood/sapwood transition has been circumvented by the use of wood cellulose as the dating material¹⁶. The only remaining non-random error which could explain the difference between the bristlecone pine and oak calibrations is the possibility¹⁶ that persistent inversion layers along the Pacific coast of the USA may have caused deviations from the global average radiocarbon level. It is not clear whether this phenomenon could account for the short-term variations observed by Suess. Similarly, within the curvilinear limits of the regression there is no clear indication of the flat spots in the calibration predicted by Ottaway and Ottaway²³.

Until the tree-ring sequences are tied to the present it will not be possible to assign calendar years to the tree-ring axis of our calibration curve. Without this it is not possible to answer questions of world uniformity of the atmospheric carbon reservoir²⁴. If world-wide uniformity is assumed then the dendrochronological age of our calibration has been shown to be within 10 yr of the range 4,100–5,240 yr b.p. (J. C. Lerman, personal communication).

Even without absolute time control there are several important conclusions that can be drawn from these results. It is clear that for the radiocarbon age span 3,600–4,550 yr b.p. the calibration is smooth within the statistical uncertainty of $\pm 3\%$ and is not significantly different from a straight line. As both the smoothness and the slope of the line are close to those statistically deduced from the bristlecone pine measurements it is reasonable to suggest that any calibration exhibiting marked deviations from this picture will ultimately be found in error. On the basis of these results it seems likely that the greater part of the calibration will tend to be smooth rather than 'wiggly'. This does not rule out, of course, the possibility of some short-term atmospheric radiocarbon variation such as that well documented for the seventeenth century AD. We cannot yet finally answer the question of whether there is a systematic difference between the bristlecone pine and Egyptian chronologies. The implication of the close agreement between the slopes of two tree-ring derived calibrations is that the deviant slope of the Egyptian calibration championed by McKerrell¹⁸ is likely to be erroneous, indicating possible inaccuracies in the Egyptian

calendar or in ¹⁴C measurements for the period before 1800 BC.

Even within the short time span so far examined there are direct implications. For example, the floating calibration covers the disappearance of pine from Irish pollen diagrams for which a close bunching of radiocarbon dates has been observed²⁵. This bunching can no longer be attributed to a 'calibration wiggle' and must, therefore, represent a synchronous event. Whether this same argument can be applied to problems outside the time range studied cannot yet be determined. If, however, most of the short-term variations disappear when a full calibration is completed, then it will ultimately reduce the uncertainty in the derivation of a true age estimate from a radiocarbon date. In fact, if it is assumed that a full calibration of the accuracy presented here is possible and if individual samples were measured with the ultimate precision of ± 12 yr, then it should be possible to produce a calibrated radiocarbon date whose 95% confidence limits fall within a bandwidth of 80 yr. This would restore radiocarbon dating to the position of the absolute dating method as was foreseen at its inception.

But the reality of the errors associated with radiocarbon dating must now be faced. Damon²⁶ and Clark¹³ pointed out that the errors based on analysis of replicated bristlecone pine samples are greater than those covered by the quoted precision. Having demonstrated the linearity of the calibration it seems possible that the variation in the dates associated with the bristlecone pine samples was due to uncontrolled errors. Since the previous measurements involving calibration samples can be assumed to be among the most careful, even these errors may well be an underestimate of those associated with routine radiocarbon measurements.

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Purification of a positive regulatory subunit from phage SP01-modified RNA polymerase

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A phage-induced subunit has been purified from phage SP01-modified Bacillus subtilis RNA polymerase. This subunit binds in vitro to RNA polymerase core from uninfected B. subtilis thereby inducing template-selective transcription and asymmetric synthesis of SP01 middle RNA.

THE interaction of virus-coded proteins with the host's RNA polymerase is an important mechanism for the control of gene expression during bacteriophage development^{1,2}. Purified RNA polymerase from *Bacillus subtilis* infected with phage SP01 or SP82 contains tightly associated, virus-coded proteins^{3–5}. A significant

property of one particular modified RNA polymerase from phage SP01-infected bacteria is that it asymmetrically transcribes hydroxymethyluracil (hmU)-containing SP01 DNA to yield the positively regulated 'middle' SP01 RNA *in vitro*. In contrast, the unmodified host RNA polymerase transcribes essentially only SP01 'early' genes³⁻⁷. The modified RNA polymerase which we previously designated as B-P is composed of the subunits of polymerase core together with two virus-coded subunits, v^{28} and v^{13} (ref. 3). (The quantity of v^{13} varies from preparation to preparation.) At least one of the two virus-coded subunits is responsible for the selective, rapid initiation of SP01 middle transcription and functions at the level of DNA binding⁸. We have previously reported⁹ the identification of a protein from a partially purified extract of SP01-infected bacteria which binds to host RNA polymerase core and converts it to the modified form. This protein has the physical properties of v^{28} . The isoelectric point (unpublished observations) and molecular weight of v^{28} identify it as identical with the peptide IV of Pero *et al.*⁴, which has been shown¹⁰ to be the product of SP01 gene 28. The product of SP01 gene 28 controls 'middle' viral gene expression¹¹. Thus, $v^{28} \equiv$ peptide IV $\equiv$ gene product 28 (gp28). We report here the purification of gp28 from RNA polymerase and show that gp28 directs the transcription of SP01 'middle' genes by *B. subtilis* RNA polymerase core enzyme.

Purification of regulatory subunit

The RNA polymerase which we used as a source of gp28 was purified from *B. subtilis* which had been infected for 10 min with

phage SP01 *sus* F4 (gene 33)-*sus* F14 (gene 34). Genes 33 and 34 code for proteins that regulate late transcription and bind to host RNA polymerase^{7,11-13}. The phage-modified RNA polymerase from *sus* F4-*sus* F14-infected bacteria is free of gp33 and gp34. It is composed of the RNA polymerase core subunits (β , β' , α , ω^1 , ω^2) and of the virus-coded subunit gp28 (data not shown).

When RNA polymerase is dissociated with 6 M urea and chromatographed on phosphocellulose in the presence of 6 M urea, the subunits of the polymerase are separated. Figure 1a shows a gel electropherogram of the fractions obtained. The α , ω^1 and ω^2 subunits (fractions 4-8) do not bind to the phosphocellulose. Elution of gp28 is reproducibly sufficiently delayed to separate it from the α , ω^1 and ω^2 subunits (fractions 10-19). The β' and β subunits bind to phosphocellulose and are eluted at higher salt concentration in 6 M urea (0.12 M and 0.27 M KCl, respectively). The pattern of subunit fractionation is similar to that previously described¹⁴. But, the RNA polymerase from *sus* F4-*sus* F14-infected *B. subtilis* lacks subunit v^{13} which would otherwise elute with gp28 and, as a result of this, gp28 is eluted in electrophoretically pure form. The fractionation of the β , β' , α , ω^1 and ω^2 subunits is also similar to that reported by Tjian and Pero¹³. Comparison of Fig. 1a and b shows a direct correlation between the activity which stimulates RNA polymerase core and the presence of gp28. The stimulating activity is manifested only in the presence of hmU-containing SP01 DNA and not with thymine-containing ϕ 29 DNA. This template selectivity is also a property of the phage-modified RNA polymerase B-P^{3,8}. The activity of gp28 can be assayed without prior removal of urea. Either gp28 is not denatured in the conditions of the phosphocellulose chromato-

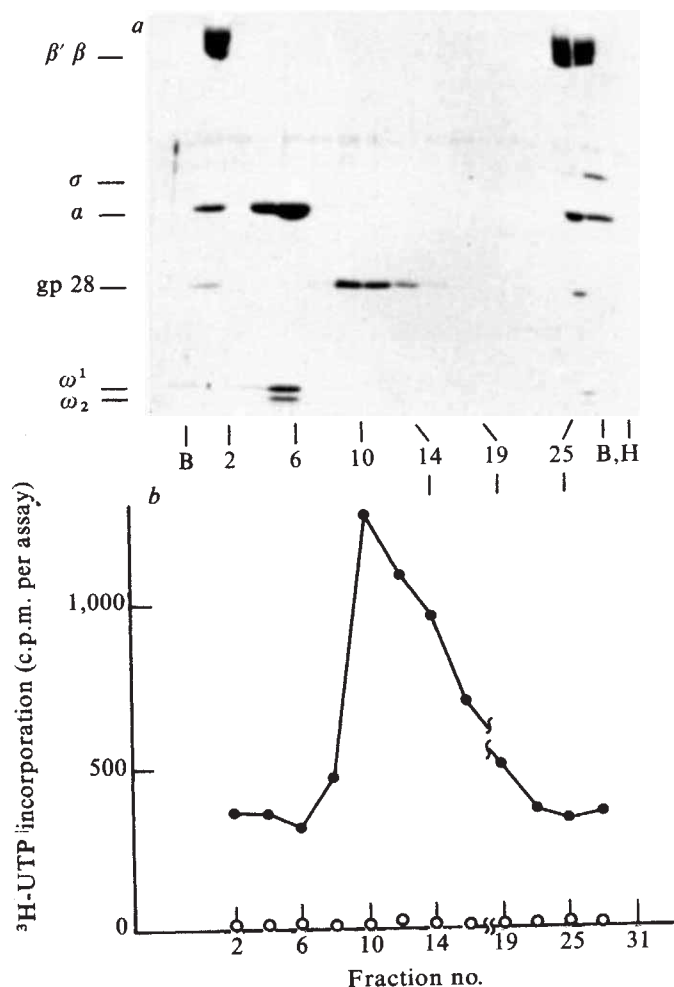


Fig. 1 Phosphocellulose chromatography of urea-treated RNA polymerase from *B. subtilis* infected for 10 min with *sus* F4-*sus* F14 SP01 phage. RNA polymerase (1.2 mg, purified according to ref. 3) was dialysed against buffer G (0.01 M Tris-HCl, pH 8, 0.1 mM EDTA, 0.01 M dithiothreitol) containing 0.05 M KCl and 6 M urea (freshly deionised) and was subsequently applied to a column of phosphocellulose (1.4 cm $\times$ 0.7 cm i.d., column volume 2 ml, rate of application 2 ml per h) pre-washed with 200 ml of the same buffer. The polymerase was allowed to equilibrate with the phosphocellulose for 30 min. The column was then washed with the same buffer at a rate of 2 ml per h, and 1 ml fractions were collected into tubes containing 0.25 ml of 50% (v/v) glycerol in buffer G plus 0.05 M KCl. *a*, Polyacrylamide slab gel electrophoresis. 100 μ l aliquots of selected fractions from the phosphocellulose column were concentrated by precipitation on ice in 10% (w/v) trichloroacetic acid. The precipitates were collected by centrifugation, washed with cold 1 M HCl and again collected by centrifugation. The samples were dried under vacuum and dissolved in the electrophoresis sample buffer which contained 3% (w/v) sodium dodecylsulphate to aid in solubilising the samples. The samples were electrophoresed in denaturing conditions (0.1% SDS) in a polyacrylamide slab gradient gel (7-15% (w/v) acrylamide) run according to Laemmli¹⁷. Marker samples were treated in an identical manner. The gels were fixed and stained in 0.2% Coomassie brilliant blue in methanol-acetic acid-water (4.5:1:4.5) and destained in methanol-acetic acid-water (10:7.5:82.5). The following samples are shown: B, 3 μ g of RNA polymerase from *B. subtilis* infected for 10 min with *sus* F4-F14 SP01 phage; numbered fractions from the phosphocellulose column; H, 3 μ g of RNA polymerase from uninfected *B. subtilis*. *b*, Assay of fractions for their ability to stimulate the activity of RNA polymerase core from uninfected *B. subtilis*. 10 μ l aliquots of fractions from the phosphocellulose column were added on ice to 0.4 μ g of RNA polymerase core in 25 μ l of TGB (0.01 M Tris-HCl pH 8, 0.1 mM EDTA, 15% (v/v) glycerol, 400 μ g ml⁻¹ bovine serum albumin). Then 4 μ g of DNA was added in 20 μ l of 0.01 M Tris-HCl pH 7.5 and 0.001 M EDTA. Each sample was then made up to 90 μ l by addition of reaction components to give the following concentration: 0.11 M Tris-HCl pH 8, 0.11 mM each of EDTA and dithiothreitol, 0.88 mM spermidine chloride, 1.1 mM each of ATP, GTP, CTP, and 0.13 mM ^3H -UTP (10.3 c.p.m. pmol⁻¹). After incubation for 10 min at 30 $^\circ\text{C}$, 10 μ l of 0.1 M MgCl₂ was added to start the reaction (the final urea concentration was 0.48 M). After 5 min the reaction was stopped and incorporation of ^3H -UTP into acid insoluble material was measured as described elsewhere³. ^3H -UTP incorporation was measured using either SP01 DNA ($\bullet$) or ϕ 29 DNA ($\circ$) as template. In the presence of RNA polymerase core and with the column fractions replaced by 10 μ l of the equivalent buffer, incorporation was 280 c.p.m. with SP01 DNA and less than 20 c.p.m. with the preparation of ϕ 29 DNA used here. Using the same conditions, 0.4 μ g *B. subtilis* holoenzyme gave 1,942 c.p.m. incorporation in the presence of SP01 DNA and 982 c.p.m. in the presence of ϕ 29 DNA. (Preparations of ϕ 29 DNA used by us previously gave appreciably more template activity with *B. subtilis* RNA polymerase core³.)

graphy, or, more probably, it renatures rapidly in the presence of polymerase core at the lower urea concentration (0.48 M) of the assay.

Fractions containing gp28 were combined and their glycerol content was increased to 25% (v/v). The gp28 was concentrated and the urea was removed by passing the combined fractions over a DEAE-cellulose column (1.8 cm × 0.4 cm i.d., 1 ml column volume) and subsequently washing the column with urea-free buffer (buffer G with 0.04 M NH₄Cl and 25% (v/v) glycerol added). A gradient of 0.04–1.0 M NH₄Cl in the same buffer was applied to the column and gp28 was eluted at 0.2 M NH₄Cl.

Properties of regulatory subunit

The activity and template selectivity of RNA polymerase reconstituted from purified gp28 and *B. subtilis* core enzyme and of the phage-modified RNA polymerase from *sus* F4-*sus* F14 phage-infected bacteria are compared in Table 1. The quantity of gp28 added to the RNA polymerase is saturating with respect to polymerising activity on SP01 DNA. The template selectivity of the gp28 is shown by the more than fourfold stimulation of RNA polymerase core in the presence of hmU-containing SP01 DNA (line 1) and by the lack of activity of thymine-containing ϕ 29 DNA (line 2). The same selectivity is observed with the RNA polymerase from *sus* F4-*sus* F14-infected bacteria (lines 3 and 4) and with gp28-containing RNA polymerase from *B. subtilis* infected for 10 min with wild type SP01 (refs 3, 8). The phage-modified polymerase from *sus* F4-*sus* F14-infected bacteria seems to be purified with near-saturating amounts of gp28 since it can be stimulated only 1.2-fold by the addition of purified gp28 (line 3). But, the maximum activity of the phage-modified RNA polymerase in the presence of saturating amounts of gp28 remains significantly less than that obtained with the combination of *B. subtilis* RNA polymerase core and gp28 (compare lines 1 and 3). It is possible that this difference is entirely due to effects introduced during the purification of these enzymes (for example, our preparations of enzyme from phage-infected cells may consistently contain a significant fraction of inactive material). But, the difference of enzyme activity may also reflect a modification of polymerase core after phage infection which affects its interaction with gp28. Such modifications are known to occur after infection with other phages (see ref. 2 for review) but have not yet been detected after phage SP01 infection.

When gp28 is combined with host RNA polymerase core, the reconstructed RNA polymerase has the ability to transcribe 'middle' SP01 genes asymmetrically *in vitro* as shown in Figs 2 and 3. ³H-RNA was synthesised for 10 min *in vitro* with excess native SP01 DNA in the presence of 0.04 M NH₄Cl and 0.16 M KCl and was analysed by hybridisation-competition with unlabelled RNA extracted from phage SP01-infected *B. subtilis*. The rationale of this analysis was described previously¹⁵. Figure 2a compares the

competition patterns of ³H-RNA made *in vitro* by *B. subtilis* polymerase core with added purified gp28 and of ³H-RNA made by the phage-modified RNA polymerase isolated with bound gp28. The similarity of these two *in vitro* synthesised RNA samples is obvious. Both are composed mainly of middle RNA transcripts as judged by the following criteria: (1) poor competition by *in vivo* early RNA; (2) maximum competition when *in vivo* middle RNA is

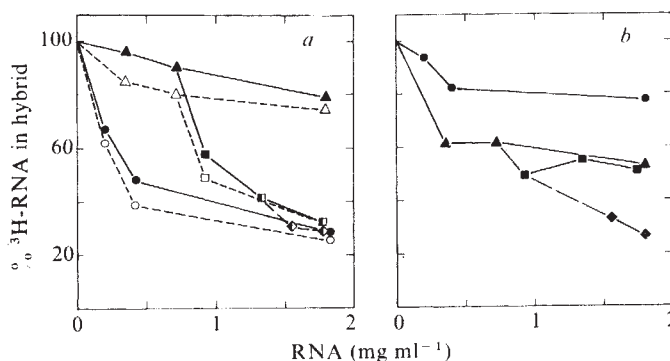


Fig. 2 Hybridisation-competition analysis of SP01 ³H-RNA synthesised *in vitro* with *B. subtilis* RNA polymerase core in absence or presence of gp28. ³H-RNA was prepared by first combining the following components in sequence on ice in 240 μ l: 50 μ l of gp28 in buffer G containing 0.2 M NH₄Cl and 25% (v/v) glycerol or 50 μ l of the same solution without gp28, 1 μ g of *B. subtilis* RNA polymerase core, 10 μ g of SP01 DNA, 1 mM each of ATP, GTP and CTP, 0.12 mM ³H-UTP (560 c.p.m. pmol⁻¹), 0.1 M Tris-HCl, pH 8, 0.1 mM each of EDTA and dithiothreitol, 0.8 mM spermidine, 0.16 M KCl. The sample was incubated for 5 min at 30 °C, and then was made 0.01 M MgCl₂ by addition of 10 μ l of 0.25 M MgCl₂. After 10 min, 25 μ l of 0.2 M EDTA and 0.2% SDS was added to stop the reaction. Synthesis of ³H-RNA by RNA polymerase from *B. subtilis* infected for 10 min with *sus* F4-*sus* F14 double mutant of SP01 phage was essentially the same except for the following changes: the final volume was 200 μ l containing 7.2 μ g SP01 DNA, 0.2 M KCl, and 0.1 mM ³H-UTP (750 c.p.m. pmol⁻¹). The ³H-RNA was extracted with phenol and prepared for analysis as described elsewhere¹⁶. The conditions of RNA synthesis used here differ from those which we used previously^{3,6} and resemble those of Losick and Pero¹⁸. We have previously assayed asymmetry of transcription at lower ionic strength, while limiting RNA synthesis to promoter-proximal segments of RNA^{3,6}. Losick and Pero¹⁸ showed *in vitro* asymmetrical transcription by modified polymerases at higher ionic strength during relatively long periods of synthesis. We compared both procedures with the phage-modified RNA polymerase B-P and found similar degrees of asymmetry as measured by RNA-RNA duplex formation. We used the conditions specified here because they yield more RNA for a given quantity of gp28. Hybridisation of the ³H-RNA (0.02 μ g ml⁻¹) to SP01 DNA (10 μ g ml⁻¹) was competed against by unlabelled RNA from SP01 infected *B. subtilis*. The aim of the competition experiments is to distinguish early, middle and late viral RNA together with their symmetrical antimessenger counterparts. Each of these three classes of messages is composed of subclasses (see ref. 16). Early (*e* and *em*) RNA is synthesised when infection is in the presence of chloramphenicol (CAM). We collected this RNA 12 min after infection at 37 °C. RNA synthesised after infection with SP01 *sus* F14 (gene 33) phage and collected 12 min after infection contains middle RNA species (*em*, *m* and *m₁*). Late RNA (*m₂* and *l*) is collected 28 min after infection with wild type phage. (The mixed competitor sequence used here allows us to neglect questions of transcriptional shut-off *in vivo*.) Δ , $\blacktriangle$, Early RNA; $\circ$, $\bullet$, middle RNA; $\square$, $\blacksquare$, mixed competition of 0.72 mg ml⁻¹ early RNA with increasing concentrations of middle RNA; $\diamond$, $\blacklozenge$, mixed competition of 0.72 mg ml⁻¹ early RNA, 0.61 mg ml⁻¹ middle RNA and increasing concentrations of late RNA. *a*, ³H-RNA synthesised with RNA polymerase core from uninfected *B. subtilis* with added gp28 (solid line, closed symbols) and ³H-RNA synthesised with phage-modified RNA polymerase from *sus* F4-*sus* F14 SP01 infected *B. subtilis* (subunit composition β , β' , α , gp28, ω^1 , ω^2 ; dashed line, open symbols). The input of ³H-RNA made with the reconstituted RNA polymerase was 927 c.p.m., and hybridisation in the absence of competing RNA was 972 c.p.m. The radioactivity bound to the filter in the absence of DNA was 14 c.p.m. The input of ³H-RNA made with the phage-modified RNA polymerase was 881 c.p.m., and hybridisation in the absence of competing RNA was 579 c.p.m. The radioactivity bound to the filter in the absence of DNA was 30 c.p.m. *b*, ³H-RNA synthesised with RNA polymerase core from uninfected *B. subtilis*. Input radioactivity was 555 c.p.m. per assay and hybridisation in the absence of competing RNA was 494 c.p.m. The radioactivity bound to the filter in the absence of DNA was 14 c.p.m.

Table 1 Activity and template selectivity of RNA polymerase reconstituted from purified gp28 and *B. subtilis* RNA polymerase core

RNA polymerase	DNA	(-)gp28	(+)gp28
1 <i>B. subtilis</i> core	SP01	811	3,458
2 <i>B. subtilis</i> core	ϕ 29	58	99
3 <i>sus</i> F4- <i>sus</i> F14	SP01	1,976	2,371
4 <i>sus</i> F4- <i>sus</i> F14	ϕ 29	93	---

The following components were first combined in sequence on ice: 5 μ l of gp28 in buffer of G with 25% (v/v) glycerol and 0.2 M NH₄Cl or 5 μ l of buffer alone, 0.1 μ g RNA polymerase in 25 μ l of TGB, 1 μ g of DNA in 10 μ l 0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA, 50 μ l of reaction components to give 90 μ l containing 0.11 M Tris-HCl, pH 8, 0.11 mM each of EDTA and dithiothreitol, 0.88 mM spermidine chloride, 1.1 mM each of ATP, GTP, CTP, and 0.13 mM ³H-UTP (100 c.p.m. pmol⁻¹). After a 5 min incubation at 30 °C, the reaction was started by adding 10 μ l of 0.1 M MgCl₂. After a further 5 min the reaction was stopped and incorporation of ³H-UTP into acid insoluble material was measured as described previously³. Incorporation for 0.1 μ g RNA polymerase holoenzyme from uninfected *B. subtilis* was 4,501 c.p.m. in the presence of SP01 DNA and 3,887 c.p.m. in the presence of ϕ 29 DNA. Incorporation in the absence of RNA polymerase core and in the presence of gp28 was 24 c.p.m.

added to early RNA; and (3) poor competition by added *in vivo* late RNA. The hybridisation-competition pattern of RNA synthesised *in vitro* with *B. subtilis* RNA polymerase core alone (Fig. 2b) is distinctly different in that all the *in vivo* RNA's compete to some extent, but poorly. The qualitative effect that gp28 has on the composition of ^3H -RNA made *in vitro* is most strikingly evident when the asymmetry of the *in vitro*-synthesised RNA is assayed in terms of the ability to form RNase-resistant RNA-RNA duplexes with early, middle and late *in vivo* RNA. Figure 3 shows that the ^3H -RNA made by RNA polymerase core alone is symmetrical (see further comments below). In contrast, the ^3H -RNA made with RNA polymerase core in the presence of added gp28 is relatively asymmetrical. More specifically, the mixed competitor assays for hybridisation-competition and the mixed RNA assays for RNA-RNA duplex formation (Figs 2 and 3) provide the following conclusions: that while the minor component of transcription from early genes is relatively symmetrical, the major component of middle RNA is almost perfectly asymmetrical. The pattern of RNA-RNA duplex formation by the RNA made with RNA polymerase from *sus* F4-*sus* F14 SP01 phage infected *B. subtilis* is identical to that for RNA made with RNA polymerase core and gp28.

Some comment on the symmetry of the SP01 RNA synthesised with *B. subtilis* core enzyme is appropriate. The data shown here imply that a slight excess of anti-messenger is synthesised (57% duplex at the highest concentration of all RNAs used, after subtracting background). This is approximately the value previously obtained in somewhat different conditions of transcription³. Approximately equal hybridisation of *in vitro* RNA to complementary DNA strands is often interpreted as indicating random transcription of helical SP01 DNA by core enzyme. While asymmetry of RNA synthesis is an excellent indication of selectivity of transcription, the reverse inference is not valid. There is as yet no clear evidence that initiation by any prokaryotic RNA polymerase core enzyme on any continuous natural DNA template is random (that is, able to use any pyrimidine nucleotide in DNA to signal initiation). Our finding of a slight excess of anti-messenger synthesised by RNA polymerase core is consistent with at least partially selective transcription. This selectivity clearly generates a collection of transcripts that are very different from *in vivo* RNA and from the transcripts generated by the fidelity of the complete transcriptases. The observed selectivity of transcription by *B. subtilis* RNA polymerase core could arise at the level of initiation or at termination.

Discussion

Thus, gp28, through its binding to RNA polymerase, is responsible for directing the asymmetrical synthesis of middle SP01 RNA *in vitro*. We surmise that gp28 confers on RNA polymerase core the template-specific ability to form rapid initiation complexes with SP01 DNA which we previously demonstrated to be a property of the phage-modified RNA polymerase⁸. How do these properties of RNA polymerase relate to the regulation of phage SP01 transcription? Fujita *et al.*¹¹ presented a model for the regulation of SP01 transcription based on an analysis of RNA synthesis during phage development¹⁶ and on the properties of SP01 mutations which affect viral transcription. The model, in its simplest form, involved a positive effector for the initiation of middle SP01 transcription, a positive effector composed of two proteins, for late SP01 RNA synthesis and a single negative element for the repression of a class of middle SP01 RNA. The products of two genes (33 and 34) which regulate the appearance of late classes of SP01 RNA have been shown to be RNA polymerase-binding proteins¹². When added to *B. subtilis* RNA polymerase core, these two subunits act synergistically to promote late SP01 RNA synthesis *in vitro*¹³. These results combined with our observations and those of Fox *et al.*¹⁰, identify the three RNA-polymerase binding proteins as two of the positive effectors proposed by Fujita *et al.*¹¹.

Although virus-coded proteins which bind to host RNA polymerase have been described^{1,2}, only those regulating SP01

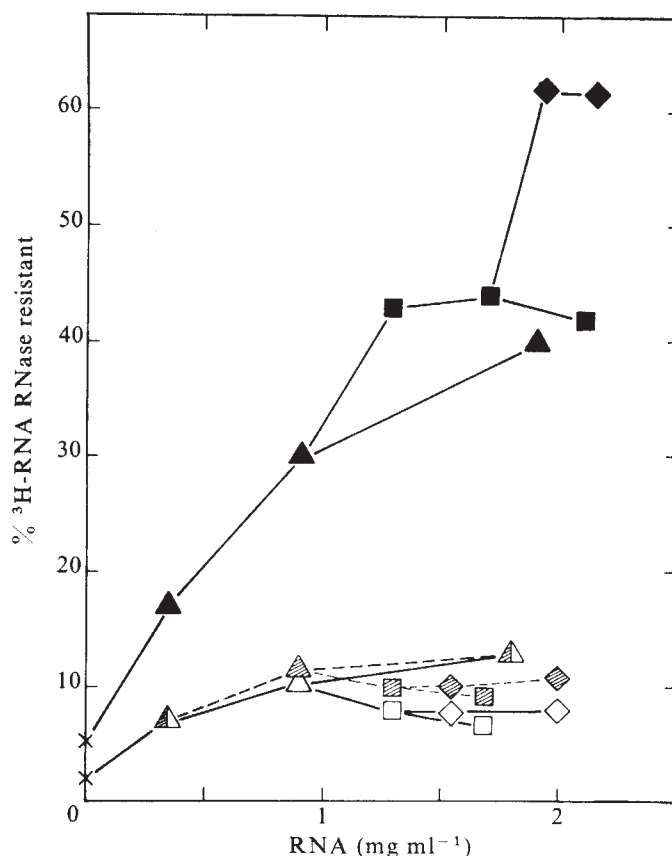


Fig. 3 RNA-RNA duplexes between *in vivo* RNA and ^3H -RNA synthesised *in vitro* with *B. subtilis* RNA polymerase core in the absence or presence of gp28 and ^3H -RNA synthesised with phage-modified RNA polymerase from *sus* F4-*sus* F14 SP01 infected *B. subtilis*. The ^3H -RNA analysed by hybridisation-competition in Fig. 2a and b was assayed for the formation of RNA-RNA duplexes with *in vivo* RNA from SP01 infected bacteria as described elsewhere^{6,19}. Each sample contained approximately $0.02 \mu\text{g ml}^{-1}$ ^3H -RNA. For ^3H -RNA synthesised by modified RNA polymerase from *B. subtilis* infected with SP01 *sus* F4-*sus* F14 (cross-hatched symbols; dashed line) and by polymerase core in the absence (filled symbols) or the presence of gp28 (open symbols) input radioactivity was 881 c.p.m., 555 c.p.m. and 927 c.p.m., respectively. The ^3H -RNA was annealed to increasing concentrations of early RNA (Δ , cross-hatched triangle, $\blacktriangle$), 0.90 mg ml^{-1} early RNA and increasing concentration of middle RNA ($\square$, cross-hatched square, $\blacksquare$), and 0.90 mg ml^{-1} early RNA, 0.80 mg ml^{-1} middle RNA with increasing concentrations of late RNA ($\diamond$, cross-hatched diamond, $\blacklozenge$).

development have up till now been amenable to *in vitro* analysis of selective transcription. The availability of these SP01-coded subunits should allow a closer study of the means by which they exert their regulatory influence. It may now be possible to study, *in vitro*, the dynamic interaction of these effectors with bacterial σ factor and with RNA polymerase core and, in that way, to understand whether these interactions alone can generate the major transitions of transcription that characterise gene expression during phage SP01 development. Models generated by such an investigation may relate to other developmental processes, at least in prokaryotes.

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Endogenous inhibitor of prostaglandin synthetase

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Mammalian serum and plasma contain an endogenous inhibitor of prostaglandin synthetase (EIPS). Human plasma fractions rich in EIPS show anti-inflammatory activity in vivo. In rats, glucocorticoids raise EIPS activity of plasma and serum. These findings suggest the existence of a natural mechanism of controlling prostaglandin synthesis, possibly related to corticosteroid action.

PROSTAGLANDINS (PGs) can elicit or intensify various unpleasant effects such as vomiting, diarrhoea, cough, pain and inflammation. Although PGs may well have an underlying defensive function, their production can at times be excessive and harmful¹. There seems, therefore, to be a need to control PG levels in tissues. It is known that these levels can be controlled through the availability of substrate for PG synthetase or the activity of enzymes metabolising prostaglandins. We describe here a new, direct endogenous mechanism of inhibiting PG synthesis that could also contribute to the control of PG levels. This was brought to light by the observation (by S.A.S.) that human serum potently inhibited the conversion of arachidonic acid into PGE₂ and PGF_{2α} by homogenate of bull seminal vesicles (BSV). Serum and plasma of other mammals also possess this property. In human plasma, this property was associated with a particular fraction, the activity of which could be increased by further fractionation. This and other evidence indicated the presence of an endogenous inhibitor of prostaglandin synthetase (EIPS). We outline below experiments on the occurrence of EIPS, on its biochemical and pharmacological properties and on its possible relationship to the mechanism of action of anti-inflammatory steroids.

Inhibition of PG synthesis by mammalian serum and plasma

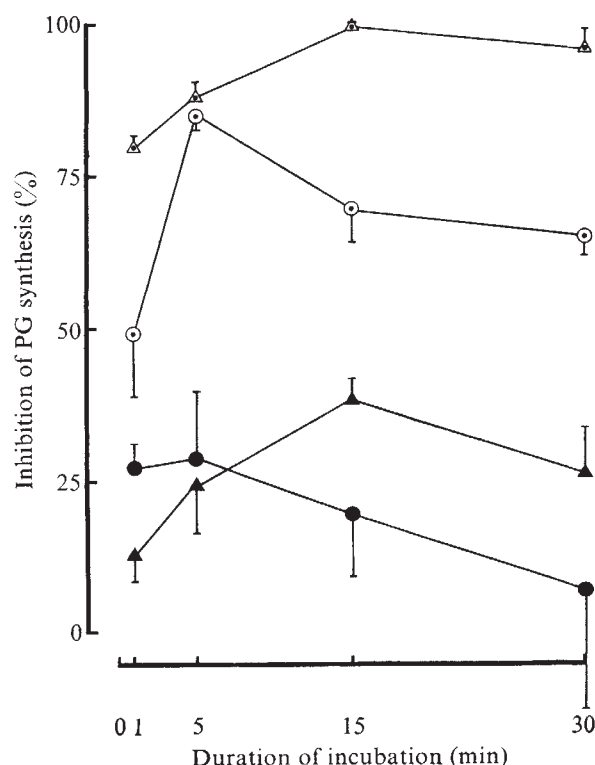
Two methods were used for measuring the extent of PG synthesis *in vitro*. First, arachidonic acid was incubated with bovine seminal vesicle homogenate and the PGs formed were either estimated as total PG-like activity on rat or hamster stomach fundus or separated by thin-layer chromatography (TLC) and estimated as PGE₂ on these tissues or as PGF_{2α} on rat colon, as previously described². Second, tritiated arachidonic acid was incubated with bovine seminal vesicle homogenate, the PGE₂ and PGF_{2α} formed were separated by TLC and estimated by liquid scintillation spectrometry. Further details of both methods are given in Tables 1 and 2.

Table 1 illustrates the concentration-response relationships for inhibition of total PG synthesis by several vertebrate sera. Except chicken, which was inactive, these sera, at concentrations of 25% v/v or less, inhibited PG production by 70% or

more. These effects were concentration-related ($P < 0.05$ for slopes of concentration-response lines). The mean values with standard error for percentage concentration (v/v) to inhibit PG production by 50% (IC₅₀) of the sera tested were: rat, $1.11 \pm 0.21\%$; human, $1.37 \pm 0.55\%$; dog, $2.96 \pm 0.3\%$; rabbit, $7.50 \pm 1.35\%$; mare, $9.30 \pm 0.41\%$.

In a few experiments, sera of guinea pig, ox and foetal calf were also tested. Guinea pig and ox sera inhibited PG synthesis at dilutions comparable with those in Table 1, but foetal calf serum was ineffective. Inhibitory activity was also found in time-expired human citrated plasma from a blood bank and in fresh rat plasma containing 0.2 mg ml^{-1} of disodium EDTA. When incubated in the standard test conditions with BSV homogenate, the IC₅₀ values of the citrated human plasma and fresh rat plasma were $0.78 \pm 0.06\%$ v/v and $1.74 \pm 0.19\%$ v/v respectively.

Fig. 1 Time course of inhibition by human serum or aspirin of PG synthesis by bovine seminal vesicle homogenate; ●, 0.5% serum v/v; ○, 5.0% serum v/v; ▲, 0.625 mM aspirin; and △, 3.13 mM aspirin. Incubations were conducted for 1, 5, 15 and 30 min. Values are the means of three experiments. Other details as in Table 1.



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Table 1 Inhibition of prostaglandin synthesis by sera

Serum concentration (%v/v)	Rat	Human	Inhibition of total PG production (%) Dog	Rabbit	Mare	Chicken
0.05	NT	13.7±5.8	NT	NT	NT	NT
0.5	27.7±4.91	34.7±13.7	14.0±4.5	2.1±1.2	15.0±9.0	NA
2.5	65.6±8.56	NT	NT	NT	NT	NT
5.0	93.3±2.14	88.7±2.2	61.0±2.1	16.8±9.0	25.0±3.0	NA
25.0	NT	90.0±3.5	97.7±0.38	91.5±0.9	91.0±1.0	NA

Inhibition values are mean $\pm$ s.e.m. NA, not active; NT, not tested. Inhibition, by pooled serum from each species, of PG production was measured in a standard assay mixture (2 ml), containing 50 mM phosphate buffer, 0.25 ml Na₂EDTA, 1.3 mM reduced glutathione, 0.5 ml bovine seminal vesicle homogenate, and an appropriate volume of the test serum or of isotonic saline as control². The reaction was started by the addition of sodium arachidonate (final concentration 61 μ M) and the tubes aerated at 37 °C with gentle shaking. After 15 min, 2 ml of 0.2 M citric acid and 16 ml ethylacetate were added to stop the reaction. After mixing and centrifugation for 5 min at 600g, 10 ml of the ethylacetate layer was evaporated to dryness, the residue was redissolved in Krebs solution and PG-like activity of the samples assayed on rat or hamster³ fundus, using PGE₂ as reference.

In some experiments, the PGE₂ and PGF_{2 α} produced by bovine seminal vesicle homogenate in the presence of various concentrations of human serum and in its absence, were separated by TLC using the AI solvent system, and assayed on rat fundus and rat colon respectively. Human serum, at 0.5, 5 and 25% v/v, inhibited production of PGE₂ by 29.7±14.9, 88.7±5.8 and 96.5±3.2% respectively. The serum at these concentrations inhibited PGF_{2 α} production by 58.0±17.1, 78.7±3.9 and 73.7±2.4% respectively. That the slope of the concentration-response line for PGF_{2 α} was not significant may be attributed to the high standard error of the estimate at 0.5% serum. In the experiments described below with labelled arachidonic acid and a purified fraction of human plasma, the slopes of the concentration-response lines were all significant ($P < 0.005$, Table 2).

The time course of inhibition of PG synthesis by human serum was compared with that of aspirin, using two concentrations of each inhibitor (Fig. 1). Human serum exerted its maximal effect between 1 and 5 min and aspirin did so between 10 and 15 min after the start of incubation.

To eliminate the possibility that these inhibitory effects might largely be due to increased prostaglandin breakdown by the test serum, we studied the effect of the most potent serum tested (rat) on the main product of PG synthesis by bovine seminal vesicle homogenate (PGE₂) in our experimental conditions. In two experiments, 0.25 μ Ci of ³H-PGE₂ (specific activity, 140 Ci per mmol) was incubated with 0.5, 5.0 or 15.0% v/v rat serum for 15 min. The reaction was stopped with citric acid, prostaglandins were extracted with ethylacetate and separated by TLC in a modified AI system². Areas corresponding to reference prostaglandins were scraped into scintillation vials and radioactivity in disintegrations per min estimated by liquid scintillation spectrometry. In the two experiments, percentage losses of tritiated PGE₂ were respectively:

0.5% serum, 0 and 30; 5% serum, 6 and 30; 15% serum, 0 and 26. In further experiments using the above conditions, the effect of various concentrations of a purified EIPS fraction of human plasma (purified Cohn IV-4, Table 2) instead of rat serum, on ³H-PGE₂ and ³H-PGF_{2 α} (specific activity 15 Ci mmol⁻¹) was investigated. In two experiments, percentage losses of tritiated PGE₂ were respectively: 50 μ g ml⁻¹ EIPS fraction, 0 and 2.4; 200 μ g ml⁻¹ EIPS fraction, 13.1 and 8.4; 500 μ g ml⁻¹ EIPS fraction 0 and 7.0. In similar experimental conditions, no loss of added ³H-PGF_{2 α} occurred. On the basis of these results we therefore concluded that the decrease in net production of prostaglandins in the presence of rat serum and purified EIPS fraction could mainly be attributed to the inhibition of PG synthesis rather than to activation of PG degradation.

Activity of human plasma fractions

The ability of partially purified Cohn fractions I to VI of human plasma to inhibit PG synthesis by BSV was tested. Fractions I, II, III, V and VI were inactive at 1,500 μ g ml⁻¹ or lower concentrations; but fraction IV (α -globulins) was active at 50, 500 and 1,500 μ g ml⁻¹. Subfractions 1 and 4 of fraction IV, which were available, were then tested at 5–1,500 μ g ml⁻¹ (Table 2). Subfraction 4 was considerably more active than subfraction 1 and was, therefore, further purified (Table 2). This purified material and various specimens of Cohn IV-4 were therefore used in the pharmacological tests described below.

Inhibition of adjuvant arthritis

As known inhibitors of PG synthetase ameliorate adjuvant arthritis in the rat^{4,5}, we tested Cohn subfraction IV-4 against the swelling of the hind feet of rats that had received an intraplantar injection of Freund's adjuvant. Treatment with Cohn IV-4 lessened the extent of both the intense swelling of the

Table 2 Inhibitory activity of human plasma Cohn fractions IV, IV-1, IV-4 and purified IV-4 against PG synthesis

Concentration (μ g ml ⁻¹)	Inhibition by (%)						
	IV	IV-1 Total PG production	IV-4	PGE ₂	Purified IV-4 PGF _{2α}	PGE ₂ *	PGF _{2α} *
50	13.4±4.6	7.8±7.5	25.0±13.0	14.7±0.82	14.3±6.4	22.4±1.0	25.2±0.91
200	NT	NT	NT	42.1±18.8	69.9±18.5	35.2±2.10	39.5±0.95
500	23.8±2.6	10.5±5.8	53.0±7.7	94.9±3.4	97.4±2.6	88.5±5.90	83.1±3.10
1,500	77.3±3.2	39.4±0.96	73.0±1.8	NT	NT	NT	NT

Inhibition values are mean $\pm$ s.e.m. Human plasma Cohn fractions IV, IV-1 and IV-4 were from Miles Laboratories. Purification of Cohn IV-4 was carried out by precipitation with 50% saturated ammonium sulphate, followed by chromatography on DEAE-cellulose, using a linear gradient of sodium acetate buffer (pH 5.0) from 0.01 to 0.5 M. Experiments with Cohn IV, IV-1 and IV-4 were performed using bioassay of total PG-like substances produced by incubating arachidonic acid with bovine seminal vesicle homogenate, as in Table 1. Experiments with purified Cohn IV-4 were performed using ³H-arachidonic acid (specific activity 80 Ci mmol⁻¹) and bovine seminal vesicle homogenate. The incubation mixtures, consisting of 1 μ Ci ³H-sodium arachidonate (0.061 mM), reduced glutathione or noradrenaline, 0.5 ml bovine seminal vesicle homogenate and purified Cohn IV-4 as indicated, in 2 ml of 50 mM phosphate buffer (pH 7.4) were incubated in glass tubes at 37 °C for 15 min with gentle shaking. The reaction was stopped by adding 2 ml 0.2 M citric acid and 16 ml ethylacetate, and 100 μ g of unlabelled PGE₂ and PGF_{2 α} were added as carriers. The radioactivity was quantitatively extracted in the ethylacetate phase, the extracts were evaporated to dryness, taken up in 400 μ l absolute ethanol and submitted to TLC on silica gel plates in the AI solvent system, with reference PGE₂ and PGF_{2 α} as markers. After chromatography, the zones corresponding to PGE₂ and PGF_{2 α} were scraped into scintillation vials and eluted with 1 ml ethanol. The radioactivity was determined by scintillation counting in Unisolve I (also containing 5 g l⁻¹ Cab-o-sil). To determine percentage inhibition of PG synthesis, radioactivity in c.p.m. of PGE₂ and PGF_{2 α} in zones from test incubates was compared with that of controls.

*Noradrenaline 1.3 mM used as cofactor instead of 1.3 mM reduced glutathione, which was used in all other incubates.

injected hind foot and the more gradual and less intense swelling of the uninjected foot (Fig. 2). Analysis of results (Mann-Whitney *U* test) on day 13 shows that, in comparison with saline, subfraction IV-4 both at 2 and 10 mg per kg body weight subcutaneously, given 1 d before adjuvant and then daily (except day 4) until day 11, significantly ($P < 0.05$) reduced the swelling of the adjuvant-treated left hind foot and of the untreated right hind foot. The higher dose (10 mg per kg) also significantly ($P < 0.05$) reduced the incidence of swelling of the fore feet and totally suppressed the incidence of nodules in the tail ($P < 0.05$).

Inhibition of bronchoconstriction

In 13 anaesthetised guinea pigs, purified Cohn subfraction IV-4, given intravenously (i.v.) in doses of 5, 20, 50 or 100 mg per kg, inhibited the increase of airway resistance and the decrease of lung compliance induced by i.v. injection of 0.1–0.5 mg per kg of arachidonate (Table 3). At doses effective in inhibiting bronchoconstriction induced by arachidonate, Cohn subfraction IV-4 did not inhibit bronchoconstriction induced by submaximal doses of $\text{PGF}_{2\alpha}$ or acetylcholine (three guinea pigs).

Like aspirin⁶, indomethacin 33 and 100 μg per kg i.v. inhibited the bronchoconstriction induced by i.v. arachidonate (Table 3), without inhibiting the response to $\text{PGF}_{2\alpha}$ or acetylcholine.

For two reasons, we also tested the purified fraction of Cohn IV-4 against bradykinin-induced bronchoconstriction in the guinea pig. First, this bronchoconstriction is known to be mainly due to stimulation by bradykinin of prostaglandin production in the lungs⁷. Second, virtually the only drugs that suppress this effect are those that inhibit prostaglandin biosynthesis⁸.

Purified subfraction IV-4 inhibited the effect of intravenous challenge with bradykinin in this preparation (Table 3). In these tests indomethacin was considerably more potent than aspirin, whereas in previous experiments of this type, indomethacin had had unexpectedly low potency compared with aspirin⁶.

In preliminary experiments on human isolated bronchial muscle, prepared as previously described⁸, Cohn subfraction

IV-4, 0.1 and 1.0 mg ml^{-1} , relaxed the muscle and inhibited the contraction induced by sodium arachidonate, 50 $\mu\text{g ml}^{-1}$.

Effect of corticosteroids on EIPS levels

In a preliminary experiment, 56 rats were given intraperitoneal (i.p.) dexamethasone sodium phosphate (0.1, 0.8 or 6 mg per kg); 4, 24, 48 and 72 h later rats were killed in groups of four or five, and the pooled serum of each group was tested for inhibitory activity. Dexamethasone raised, in a dose-related way, the ability of the serum to inhibit PG synthesis. This effect was apparent at 24 h after treatment, it reached a peak at 48 h and was considerably diminished at 72 h. In a similar experiment, using 72 rats, an i.p. dose of hydrocortisone sodium succinate (11, 90 or 360 mg per kg) likewise raised the inhibitory activity of the serum, with a comparable time course. In a third experiment, on 20 rats, aldosterone (0.2 mg per kg) failed to increase the inhibitory activity of the serum over a 72-h period.

In a further experiment on rats, dexamethasone sodium phosphate (24 mg per kg) or hydrocortisone sodium succinate (360 mg per kg) were given i.p. and 4, 24, 48 and 72 h later, animals were killed in groups of five and each bled individually from the dorsal aorta into tubes containing 0.2 mg ml^{-1} of disodium EDTA as an anticoagulant. The plasma was separated by centrifugation for 15 min at 600g and the potency in inhibiting PG synthetase (EIPS level) of each plasma sample determined by the standard test procedure using bovine seminal vesicle homogenate (details as in Table 1). The results obtained showed that both dexamethasone and hydrocortisone raised plasma EIPS levels with comparable time courses (Fig. 3).

Endogenous inhibitor of PG synthetase

We have presented evidence that incubation of mammalian blood plasma or serum with bovine seminal vesicle homogenate results in a lowered production of prostaglandins. There are several possible ways in which this could occur, but most of these are incompatible with the evidence. The effect cannot be due to inhibition of phospholipase A because (1) EDTA, which inhibits phospholipase-A, is present in the assay medium and (2), an optimal amount of sodium arachidonate was added to the medium. Neither can the effect be due to inhibition of

Table 3 Inhibition of increased airway resistance and decreased lung compliance induced by sodium arachidonate or bradykinin

Treatment	Dose (mg per kg)	Challenge substance	% Inhibition of response to challenge*	
			Resistance	Compliance
Purified Cohn IV-4	5	Arachidonate sodium	31.8 $\pm$ 4.7	24.4 $\pm$ 5.3
	20		44.3 $\pm$ 6.7	34.3 $\pm$ 7.4
	50		67.7 $\pm$ 0.3	49.0 $\pm$ 3.2
	100		67.0 $\pm$ 8.0	64.0 $\pm$ 0
	5	Bradykinin	34.7 $\pm$ 1.7	19.5 $\pm$ 5.5
	20		48.3 $\pm$ 4.4	33.4 $\pm$ 11.8
	50		67.0 $\pm$ 0	73.2 $\pm$ 19.8
	100		94.0 $\pm$ 6.0	86.5 $\pm$ 13.5
Aspirin sodium	2	Arachidonate sodium	85.5 $\pm$ 3.5	71.5 $\pm$ 6.5
	2		82.0 $\pm$ 9.6	80.7 $\pm$ 9.9
Indomethacin sodium	0.03	Arachidonate sodium	47.0 $\pm$ 15.8	58.7 $\pm$ 15.7
	0.1		90.5 $\pm$ 9.5	85.5 $\pm$ 14.5
	0.01	Bradykinin	35.5 $\pm$ 9.9	25.3 $\pm$ 8.2
	0.03		58.0 $\pm$ 14.0	53.0 $\pm$ 3.0
	0.1		88.5 $\pm$ 11.5	86.0 $\pm$ 6.0

Male albino guinea pigs (400–600 g, Dunkin Hartley strain) were anaesthetised with pentobarbitone sodium, 60 mg per kg i.p. The trachea was cannulated for artificial ventilation by a Starling pump (5–8 ml at 72 strokes per min) by a Fleisch 0000 pneumotachograph. The difference in pressure across this device was measured with a Grass differential pressure transducer. This gives a flow signal that was fed into a Hewlett-Packard 8816A analogue computer. Transpulmonary pressure (TPP) was measured with another differential pressure transducer via side-arms to the thoracic cavity and the tracheal cannula. The signals for flow and TPP were applied to the analogue computer so that there was approximately 180° phase difference. Dynamic compliance and airway resistance were derived by the computer from the ingoing signals. Drugs were administered intravenously through the external jugular vein. Arachidonic acid, 99% pure from Sigma was given in doses of 0.1–0.5 mg per kg as the sodium salt. Bradykinin as triacetate from Sigma was given at 1–5 $\mu\text{g per kg}$. Test drugs were injected 45 s before challenge. Other details as in Table 2.

*Values are mean $\pm$ s.e.m.

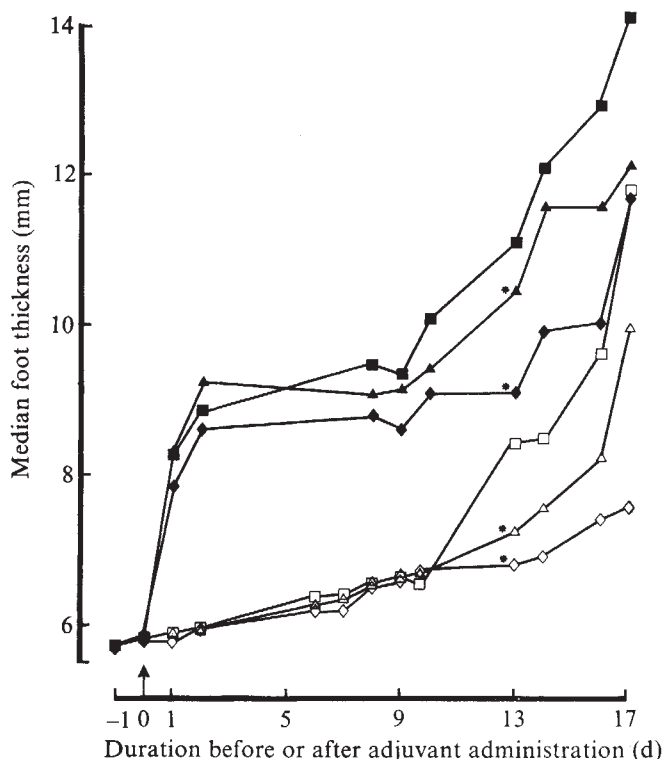


Fig. 2 Inhibition by Cohn subfraction IV-4 of adjuvant arthritis in the rat, induced by the method of Newbould⁴. A suspension of 25 μ g of killed, ground *Mycobacterium butyricum* (Difco) in 50 μ l of light liquid paraffin was injected into the plantar surface of the left hind foot of each of three groups of 12 rats on day 0 (arrow). On days -1 to 3 and days 5 to 11, saline (■) or subfraction IV-4, 2 mg per kg (▲) or 10 mg per kg (◆) was injected subcutaneously. Foot swelling was measured daily by micrometer, placed sagittally. Closed symbols, injected foot; open symbols, uninjected foot. *, $P < 0.05$ for difference of test from saline-treated controls (Mann-Whitney U test).

PG release from intact cells, because the cells of bovine seminal vesicle homogenate have already been disrupted. The experiments with tritiated PGE_2 and $\text{PGF}_{2\alpha}$ eliminate the possibility that the effect of serum and a purified fraction of plasma rich in EIPS activity was due to activation of enzymes destroying prostaglandins. We conclude that mammalian blood plasma and serum contain a mechanism for inhibiting PG synthetase. The presence of this mechanism in plasma indicates that it is not simply an artefact generated in serum during blood clotting. Since PG synthesis can be inhibited by antioxidants^{9,10}, one of which (propyl gallate) has anti-inflammatory activity *in vivo*¹⁰ and since macromolecules and macromolecular complexes have an undoubted, though little-understood, capacity to act as free radical stabilisers^{11,12}, we have considered the possibility that the inhibitory effect might be a nonspecific property of blood proteins. This possibility was eliminated by the finding that this effect of plasma was restricted to the α -globulin fraction, representing about 5% of total plasma proteins. We therefore postulate that plasma and serum contain an endogenous factor (or factors) that potently inhibits PG synthetase (EIPS). Analysis of its biochemical properties showed EIPS to be a protein, because neither deproteinised plasma nor serum inhibited PG synthesis and because EIPS was inactivated by Pronase. EIPS could not be dialysed through a Cellophane membrane, indicating a large molecular size. Further characterisation of human plasma EIPS has shown that its activity is associated with haptoglobins (in preparation). Smith *et al.*¹³ have previously described an anti-inflammatory fraction of human plasma. This differs in two main ways from EIPS, however. First, this fraction does not inhibit prostaglandin synthesis; second, it is of relatively low molecular weight (about 1,000).

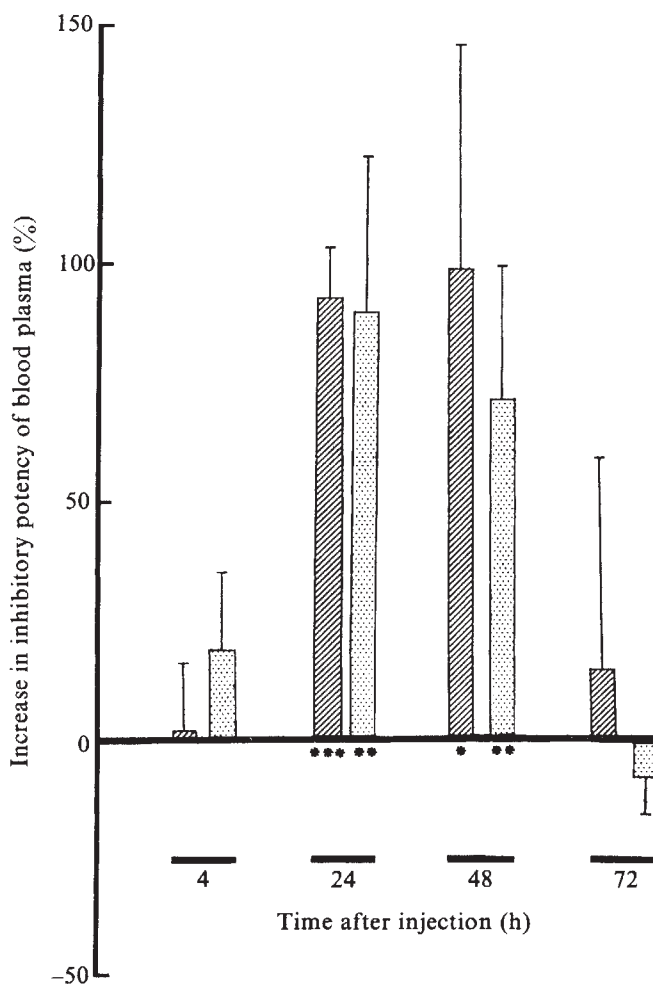
These observations raise the question: does EIPS represent a natural mechanism for control of PG synthesis? If so, how is EIPS inactivated when a need for PG synthesis arises? If so, again, what is the relevance of EIPS to diseases associated with excessive or unbalanced PG production?

Anti-inflammatory drugs

By analogy with morphine, which may be regarded as a mimic of an endogenous ligand of the opiate receptor^{14,15}, the possibility arises that non-steroidal anti-inflammatory drugs, which inhibit PG synthetase, may act as mimics of EIPS. Other relationships between these drugs and EIPS are possible, however.

Several studies have shown that corticosteroids interfere with the release or production of prostaglandins or related substances in preparations that include human skin homogenate¹⁶, dog hind leg¹⁷, adipose tissue¹⁸, mesenteric artery¹⁹, cultured synovial cells^{20,21}, cultured fibrosarcoma cells²², and isolated guinea-pig lungs in anaphylaxis²³. In most of these studies the mechanism by which corticosteroids acted was not fully identified; but Nijkamp *et al.*²³ showed that corticosteroids, with a relative potency comparable to their anti-inflammatory activity *in vivo*, interfere with the release of arachidonate, induced by rabbit aorta contracting substance-releasing factor (RCS-RF).

Fig. 3 Changes in EIPS levels of blood plasma taken from rats at various times after the intraperitoneal injection of dexamethasone or hydrocortisone. Cross-hatched column, 360 mg per kg hydrocortisone sodium succinate; stippled column, 24 mg per kg dexamethasone sodium phosphate. Each column represents the mean $\pm$ s.e.m. of five determinations. For other details see text and Table 1. *, $P < 0.05$; **, $P < 0.025$; ***, $P < 0.0005$ for difference from saline-treated controls (Student's t test).



In the experiments described here we have shown that, in normal rats, administration of a natural or a synthetic anti-inflammatory glucocorticoid (hydrocortisone or dexamethasone), but not of the mineralocorticoid, aldosterone, increased the ability of the plasma or serum to inhibit prostaglandin synthesis by bull seminal vesicle homogenate. This effect is presumably due to the increase of EIPS. This suggests that glucocorticoids may act, at least in part, by increasing EIPS.

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letters to nature

A search for the reported 400-keV γ -ray line from Crab Nebula

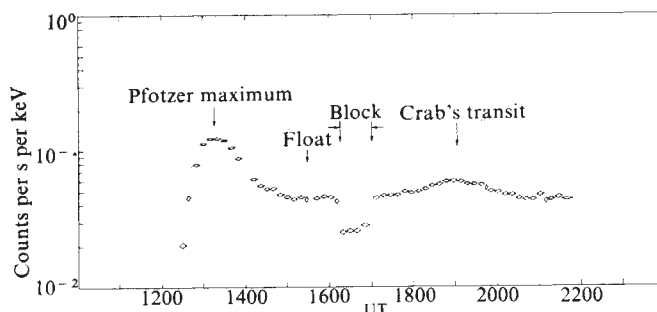
LEVENTHAL *et al.*¹ have reported a possible γ -ray line at 400 ± 1 keV from the Crab Nebula. The measurement was achieved with a balloon-borne high resolution γ -ray telescope flown from Alamogordo, New Mexico on 10–11 May 1976. The intensity and the width of the line are reported to be $(2.24 \pm 0.65) \times 10^{-3}$ photon $\text{cm}^{-2} \text{s}^{-1}$ and $\lesssim 3$ KeV, respectively. These authors suggested that a possible origin of this line may be the gravitational redshift of the 0.511 MeV annihilation line produced near the surface of the neutron star. The line, if real, is of great astrophysical interest, and a confirmation of the measurement is therefore important. We report here the results of our search for this feature.

On 10 June 1974 we conducted a balloon experiment over Palestine, Texas with a large volume high resolution γ -ray spectrometer. The instrument which has been described previously² consisted of four 40-cm³ Ge(Li) crystals (only three were operational for the flight) operating in the energy range of 0.06–10 MeV. This cluster of detectors is surrounded by a CsI(Na) anticoincidence shield for reducing the background and providing collimation of 25° FWHM at 400 keV. The total effective area (efficiency $\times$ front area) is 10.4 cm² and the energy resolution is 2.2 keV FWHM at the same energy. The objectives of the experiment were to measure (1) X-ray and γ -ray emissions from the Crab Nebula, (2) diffuse cosmic fluxes (3) atmospheric γ rays and (4) the instrument's background in a space environment. A study of the 0.511 MeV γ rays from the terrestrial and extraterrestrial environments, results of the observation of a 'flare-like' γ -ray line event, and the hard X-ray spectrum (53–300 keV) of the Crab Nebula are reported elsewhere^{3–5}. A detailed analysis of the γ -ray spectrum (0.3–10 MeV) of the Crab Nebula apart from the 400 keV and 511 keV lines is now underway, and will be reported in the future.

The balloon was launched at 1204 UT and reached a float altitude of 2.9 g cm⁻² at approximately 1530 UT where it remained for 6.5 h before cutdown. During the entire flight, the azimuth servo system maintained the instrument's

aperture in a southerly direction and the elevation was set 10° from the zenith, allowing the Crab Nebula and the Sun, separated by 4°, to transit directly through the field of view. Since the viewing axis azimuth and zenith angles were fixed, background variations which may be associated with changes in these parameters by pointing the instrument towards and away from the target such as the method used by Leventhal *et al.*¹ are therefore eliminated. Background variations owing to the balloon spatial drifts are estimated to be $\sim 1\%$. Figure 1 shows the time history of the counting rate measured by the three Ge(Li) detectors in the range 65–100 keV. During the first part of float, the aperture was blocked with a 20.3 cm diameter $\times$ 10.2 cm thick NaI(Tl) crystal also in anticoincidence with the prime sensors and the rate decreased from 1.6 counts per s to 0.9 counts per s. The difference can be attributed to γ rays originating from the atmospheric and diffuse cosmic components and entering through the aperture. The Sun and the Crab Nebula transited through the field of view at 1845 UT and 1902 UT, respectively. By 1902 UT the rate had increased to 27% over

Fig. 1 Time history of the counting rate measured by three of the four Ge(Li) detectors (65–100 keV). During the first part of float at 2.9 g cm⁻², the aperture was covered by a 20.3 cm diameter $\times$ 10.2 cm thick NaI(Tl) detector and the rate decreased from 1.6 to 0.9 counts per s. The difference can be attributed to aperture fluxes which consist of the atmospheric and diffuse cosmic X-rays. The Crab Nebula transited directly over the field of view at 1902 UT.



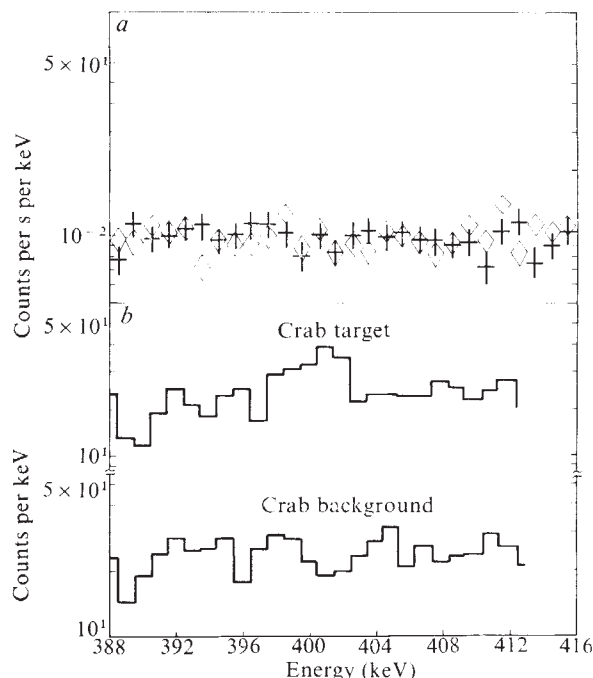


Fig. 2 Energy loss spectra. Our measured rates around 400 keV (a) are compared with those reported by Leventhal *et al.*¹ during live-time intervals of 7,113-s (Crab target) and 7,084-s (Crab background), respectively (C. J. MacCallum, personal communication). Their measured line flux of 2.24×10^{-3} photons $\text{cm}^{-2} \text{s}^{-1}$ should increase our measured rate in the 398–403 keV bin of the Crab plus Background spectrum by 1.26×10^{-2} counts per s. Such an increase of 3.9σ statistical significance is not observed in our data. In a +, Crab plus background, 104 min; ◇, background, 128 min.

the background measured before and after the transits. Since the Solar Geological data (No. 360–Part 1, 1974) indicated no significant X-ray and γ -ray activity during this period, contributions to the enhancement of the measured rate from the Sun are expected to be negligible.

Figure 2a shows two energy-loss spectra in the range 388–412 keV with one of the spectra accumulated over a 104 min (1812–1956 UT) live-time interval during the Crab Nebula transit and the other over a 128 min live-time interval before (1702–1742 UT) and after (2016–2148 UT) the transit. The length of the source transit-interval selected for analysis was chosen such that the ratio of the total number of counts from the Crab Nebula over its statistical significance was maximised. The net average exposed effective area of the instrument to the Crab Nebula during the transit was 5.62 cm^2 at 400 keV. This value is determined experimentally using laboratory radioactive sources and computation with the Monte Carlo technique^{3,6} and further corrected for effects of attenuation of the overlying atmosphere and materials in the gondola, balloon spatial drift³ and leakages through the shield. Also shown in Fig. 2 for comparison are the corresponding spectra measured by Leventhal *et al.*¹ (C. J. MacCallum, personal communication). During the Crab Nebula transit the difference in our data of the counting rate measured in the 398–403 keV bin and the average rate measured in the two adjacent bins 388–398 keV and 403–413 keV is $(-2.85 \pm 3.27) \times 10^{-3}$ counts per s. The null result is different from the measurement of Leventhal *et al.* of a positive increase of 4.0σ significance using the same bin selection. Their reported line flux of 2.24×10^{-3} photons $\text{cm}^{-2} \text{s}^{-1}$ from the Crab Nebula would increase our measured rate in the 398–403 keV bin of the Crab plus background spectrum by 1.26×10^{-2} counts per s. Such an increase of 3.9σ statistical significance is clearly not observed in our data. In addition, our background spectrum with the Crab Nebula outside the field

of view shows rates in the same three energy bins similar to those accumulated during the source transit. As these results are also consistent with our prediction of background variation of 1% owing to balloon spatial drifts, we may rule out the possible explanation that our null results in observing the 400 keV line is caused by systematic background effects.

In conclusion, we did not observe the 400-keV γ -ray line from the Crab Nebula on 10 June 1974. Our result is in contradiction with Leventhal's measurement if a constant source intensity is assumed. The two observations were separated by 2 yr, however, and we cannot rule out the possibility that the feature may vary with time. Observations with a high resolution instrument may resolve this issue.

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Hadronic photoabsorption and pair production in pulsars

THERE are two existing models for the acceleration of positive charged particles near the magnetic polar caps of a pulsar. In the first, the work function for a ^{56}Fe ion bound in the lattice at the stellar surface is assumed, following exhaustive calculations of Flowers *et al.*¹, to be too large for the occurrence of ion emission. Electron-positron pairs are created through magnetic conversion of curvature photons²; the electrons are accelerated inwards to the stellar surface, and the positrons outwards along open lines of magnetic flux to the light cylinder. In the second, the work function is assumed to be negligible and ion emission limited only by space charge effects. In the corotating frame of reference, all components of the surface electric field are zero and inertial ion acceleration^{3–5} occurs. For surface magnetic flux densities $B \gtrsim 10^{12} \text{ G}$, we shall attempt to show here that the nature and mode of acceleration of the plasma moving outwards along open magnetic flux lines are determined, not by these processes, but by the occurrence of hadronic photoabsorption reactions in the electromagnetic showers produced by ultra-relativistic electrons incident on the stellar surface. This work was prompted by the comment⁴ that magnetic conversion of backward moving photons from these showers may be an important source of pairs. The consequences of hadronic photoabsorption do not seem to have been considered previously. Here, it is shown that photons of the energy necessary for conversion in a magnetic flux density of 10^{12} G are emitted with high probability in hadronic photoabsorption reactions, and an equation is obtained for the acceleration potential difference in a one-dimensional model of electron-positron pair creation at the magnetic polar cap. The properties of the accelerated plasma are important owing to its probable

connection with radio emission and with the phenomenon of subpulse drift occurring in certain pulsars (see, for example, refs 6, 7).

Consider a photon emitted, for example, at an angle of 150° to the direction of the primary electron, assumed parallel with **B**. For $B = 10^{12}$ G, the mean free path for magnetic conversion⁸ is in the interval $1 \leq \lambda \leq 10^4$ cm for photon energy ϵ such that $9 \geq \epsilon \geq 5$ MeV. A photon of these properties would have a negligible probability of creation by purely electromagnetic processes, as shown by the shower distribution function tables of Messel and Crawford⁹.

The transverse momentum of most photons in the shower is $\lesssim 10 mc$, defined with respect to **B**, and therefore the properties of the shower should be unchanged by the magnetic flux. From the shower distributions⁹ (for air) the photon track length at energies of more than 300 MeV is estimated to be $1.6 W$ radiation lengths, where W is the primary electron energy in GeV. For $W \approx 10^3$ GeV, several photoabsorption reactions would occur per shower. The equilibrium state of matter at the stellar surface would be determined by the formation of light nuclei (with negligible work functions) in the spallation of ^{56}Fe and their emission from the surface by thermal excitation. At the temperatures and densities considered, fusion and diffusion would seem unimportant. Owing to the spallation and the interaction-induced disorder, the matter would have the characteristics of a glassy solid. However large the ^{56}Fe work function, the plasma moving outwards must have an ionic component. The charge spectrum of light nuclei accelerated would include $Z = 3-5$, normally considered to be present in galactic cosmic rays as a result of the interaction of heavier nuclei with the interstellar medium.

For photon energies below the meson threshold, the most important photoabsorption reaction is the formation of the giant dipole resonance. The cross section, integrated over photon energy from 15 to 30 MeV, is a large fraction of the classical sum rule result¹⁰, $60(NZ/A) \text{ mb MeV}$. For mass numbers $A > 40$, neutron emission is the dominant decay mode of the resonance. Proton emission occurs, but is less probable. Photons with energy in the interval $5 < \epsilon < 9$ MeV are produced with approximately unit probability in both the further decay of the residual nucleus and the capture of the neutron. The average number of photons from these processes entering the magnetosphere with energy such that their mean free path for magnetic conversion is in the interval $1 < \lambda < 10^4$ cm can be estimated, from the shower distributions⁹, to be of the order of 1-10 for a primary electron of 10^3 GeV. A laboratory measurement of the number and energy spectrum should be possible at the lower energies of existing electron beams.

We later summarise some results for a one-dimensional model of pair creation by this mechanism. It is assumed that acceleration occurs for $0 < z < z_a$, where z is the distance from the stellar surface measured along an open line of magnetic flux at the polar cap, and $z_a \lesssim 10^4$ cm. In the corotating frame of reference, the potential $\phi(z)$ satisfies the condition

$$\phi(0) = \left(\frac{\partial \phi}{\partial z} \right)_{z_a} = 0 \quad (1)$$

In units of $\sigma_0 c$, where σ_0 is the (positive) corotational charge density¹¹, the ion current density is α_1 , and the electron-positron current density resulting from magnetic conversion between z_0 and z_a is α_2 . The lower limit $z_0 \ll z_a$ represents a maximum photon energy. It is assumed that $\alpha_1 + \alpha_2 = \alpha \leq 1$.

The first result obtained is that the mean number of photon conversions per primary electron and unit interval of z is of the form

$$G(z) \propto z^{-1}, \quad z_0 < z < z_a \quad (2)$$

as a function of z . This is independent of the photon angular distribution, is exact if the photon energy spectrum is $f(\epsilon) \propto \epsilon^{-2}$,

and is a satisfactory approximation for any reasonable $f(\epsilon)$. It is assumed, on the basis of the discussion of the giant dipole resonance, that the form of $f(\epsilon)$ (though not its scale) is independent of primary electron energy. With this result, the potential $\phi(z)$ satisfying the boundary conditions (1) can be found from Poisson's equation, assuming all particle velocities equal to c . The maximum acceleration potential is

$$\phi(z_a) = -2\pi\sigma_0 z_a^2 (1 - \alpha - \alpha_2 / \ln(z_a/z_0)) \quad (3)$$

Following the discussion of hadronic photoabsorption, we assume that the mean number of photon conversions between z_c and z_a per electron created at z and accelerated inwards to the stellar surface is

$$F(z) = C\phi(z), \quad z_c < z < z_a \\ = 0, \quad z < z_c \quad (4)$$

where the lower limit z_c satisfies $z_0 \ll z_c \ll z_a$ and C is a function of this lower limit and of B . The equation for $\phi(z_a)$ can be derived from the condition for a time-independent electron-positron current density. It is

$$4\phi(z_a)/\phi(z_c) = \exp(2F(z_a)), \text{ in the limit } \alpha \rightarrow 1 \quad (5)$$

or

$$2\phi(z_a)/\phi(z_c) = \exp(\frac{2}{3}F(z_a)), \quad 1 - \alpha \gg \alpha_2 / \ln(z_a/z_0) \quad (6)$$

The solutions of equations (5) and (6) are insensitive to z_c and as functions of C are approximately $\phi(z_a) \propto C^{-1}$. We estimate $\phi(z_a) \approx 10^3$ GeV for $B = 10^{12}$ G on the basis of the earlier (order of magnitude) discussion of photon production. Thus pair production and the outward flow of positrons may occur through this mechanism rather than the conversion of curvature radiation which would require a small radius of curvature ($< 10^7$ cm) for the open magnetic flux lines at the stellar surface. Both a laboratory measurement of photon production and a detailed study of the effect of the magnetic flux on shower properties, however, are necessary for quantitative estimates of C and $\phi(z_a)$.

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A classification scheme for pulsars

WE have already¹ distinguished between three types of pulsars depending upon their mode of formation. Here we discuss some implications of this classification scheme. We suggest the nomenclature types L, S and D for the three types of pulsars. Type S (S for single) consists of pulsars formed as a result of supernova explosions of single stars. Types D and L refer to pulsars which come from massive close binary systems. Pulsars produced by

supernova explosions in and leading to the disruption of the binaries belong to type D (D for disrupted), while the old, rejuvenated neutron stars liberated from binaries by the supernova explosions of the companion stars constitute type L (L for liberated). Type D consists of two subsets; a pulsar belongs to type D₁ if its formation, accompanied by the disruption of the binary, is a result of the supernova explosion of the first (originally the more massive) star; to type D₂, if a result of the supernova explosion of the second (originally the less massive) star. Thus type D₁ pulsars are associated with OB runaways, while type D₂ pulsars are associated with type L pulsars.

This distinction between the three types of pulsars has two significant implications. First, the age of a type L pulsar is not simply determinable from its spin-down rate. Pulsars in massive close binaries are born in rapid rotation like single pulsars², but slow down rapidly³. The neutron star spins up due to mass accretion from the companion star and mass loss from the system⁴. In terms of the concept of spin-down age, the neutron star ages rapidly immediately after its formation but is rejuvenated later. Only if the type L pulsar is spinning as rapidly at the time of the disruption of binary, as it was at the time of its formation, is its spin-down age relevant. For type D pulsars, of course, it is as good a measure of the true age as it is for type S pulsars.

Second, a type L or a type D₂ pulsar cannot be assumed to have always been moving with its present velocity, which is the orbital velocity the pulsar had at the time of the disruption of the binary by the second supernova explosion. Before that the system was moving with the velocity it had acquired as a result of the first supernova explosion. An estimate of the velocity can be made from the galactic z -distribution of massive X-ray binaries—a stage reached by the OB binary some 4–5 Myr after the first supernova explosion⁵, which can be assumed to have taken place in the galactic plane. As the average distance of massive X-ray binaries above the galactic plane is ~ 90 pc (ref. 6), we conclude that the centre of mass of the system is accelerated to a z -velocity of ~ 20 – 30 km s⁻¹ by the first supernova explosion.

Since the time interval between the first and the second supernova explosion is about 5–7 Myr (ref. 5), the binary would have moved a distance 100–200 pc perpendicular to the galactic plane before being disrupted by the second supernova explosion. Thus while type S and type D₁ pulsars are born close to the galactic plane, type D₂ and L pulsars are born at large $|z|$ values. If, however, the space velocity of the pulsar at the time of the disruption of the progenitor binary had a z -component, the present z -position of the pulsar could be above or below the z -value at which the pulsar was formed. Further, since the space velocities of type L and type D pulsars are typically an order of magnitude larger than the centre of mass velocities of their progenitor binaries, a pulsar could move a substantial distance in the z -direction after the disruption of the binary so that although it was formed at a large $|z|$ value, it could now be close to the galactic plane. It could even have crossed the galactic plane and may now appear on the other side. Thus while a type L or a type D₂ pulsar is born at $|z| \sim 100$ – 200 pc, its present distance from the galactic plane would depend upon the magnitude and the sign of the z -component of its space velocity and on its age as a single object.

We can now review the galactic z -distribution of some pulsars. PSR 1133 + 16 has a transverse velocity of 320 km s⁻¹ at a height $z = 150$ pc above the galactic plane⁷ and a spin down age of 5 Myr. According to ref. 1 it is a type L pulsar. Two hypotheses can then explain its present z -position. (1) The progenitor binary had moved a z -distance of about 150 pc between the first and the second supernova explosions so that the pulsar was formed at a height ~ 150 pc above the galactic plane and is now moving parallel to it. In that case its radial velocity should be ~ 120 km s⁻¹. (2) Or, the pulsar was born ~ 150 pc below the galactic plane. The orbital plane of the progenitor binary was suitably inclined to the galactic plane to give the liberated pulsar an appropriate z -velocity. Some time ago, the pulsar must have crossed the galactic plane and is moving up. A similar explanation holds for PSR2021 + 51 which has a transverse velocity $\lesssim 400$ km s⁻¹ but is only 120 pc above the galactic plane⁷. PSR 0823 + 26, PSR0834 + 06 and

PSR1237 + 25 are respectively 420, 210 and 370 pc above the galactic plane and have transverse velocities 506, 118 and 178 km s⁻¹ respectively⁸. They are all type L pulsars¹. They must have been born ~ 150 pc above the galactic plane and are moving up.

PSR1929 + 10 is located at $z = -7$ pc and has a velocity 75 km s⁻¹ perpendicular to the galactic plane⁷. Since its velocity is much too small for its status as a type L pulsar¹ it must have a large radial velocity. The present location of the pulsar can be explained if we assume that it was born ~ 150 pc below the galactic plane and is now on its way up. PSR0329 + 54 has a transverse velocity of $\lesssim 450$ km s⁻¹ and is located at $z = -28$ pc (ref. 7). We have classified it as a type D pulsar¹. Either it is a type D₁ pulsar born at this z -value and is moving parallel to the galactic plane, or, alternatively, it is a type D₂ pulsar which was born at a large z -value and has since moved closer to the galactic plane. PSR0950 + 08 has a transverse velocity of 50 km s⁻¹ at $z = 71$ pc (ref. 7). Since it is a type D pulsar¹, it must have a large radial velocity. Further to explain its low z -value, in spite of its large age (17 Myr), it must be postulated that the pulsar was born at this z -value and is moving parallel to the galactic plane. The Vela pulsar 0833 – 45 is at a low value of z (-24 pc) as a young type S pulsar should be. Another type S pulsar, PSR2016 + 28, is also close to the galactic plane ($z = -33$ pc)⁸. But since it is very old (59 Myr) it must be moving parallel to the galactic plane. The Crab pulsar is located 200 pc below the galactic plane. Since it is an extremely young type S pulsar¹, it has not moved much from its place of birth. Therefore its progenitor could not have been an OB star, all of which are confined to the galactic plane. Thus, Crab pulsar must be the result of the supernova explosion of a late type, low mass star.

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Deuterium enrichment in interstellar HCN and HNC

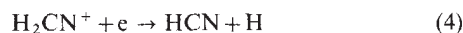
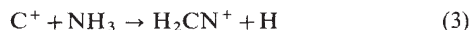
THE cosmic abundance of deuterium, expressed as the ratio D/H, is thought to be approximately 2×10^{-5} (ref. 1). Observations on deuterium-containing molecules, such as DCN (refs 2,3) and DNC (ref. 4), however, indicate much higher values for the molecular ratios DCN/HCN and DNC/HNC. In the case of DCN this was attributed to chemical enrichment involving equilibria such as⁵



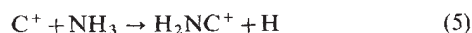
or to other mechanisms⁶. But it is difficult to reconcile the observed enrichment of DNC with any of these suggestions. Here we explore the alternatives and correct previous suggestions about the mechanism of formation of interstellar HCN. We consider conditions appropriate to dense molecular clouds such as that in Ori A or NGC2264 where the visual/ultraviolet optical depth is very high and ionisation is predominantly by cosmic rays.

For such clouds ion-molecule reactions in the gas phase^{7,8} seem to account satisfactorily for many of the observations on molecular species and we shall base our discussion on this theme. Reactions on dust grains, especially very small ones, may also

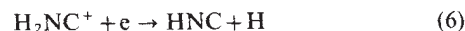
play a significant part⁹. In it, the formation of HCN is attributed to the following reactions:



In the low temperature conditions that prevail in interstellar molecular clouds, however, the N–H bonds will not be disrupted in equation (3) and so it should be written

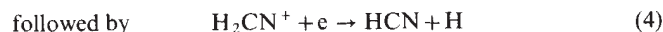
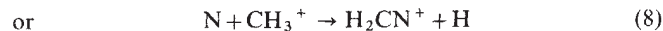
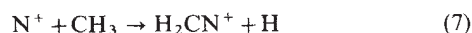


which would be followed by



Thus the ion–molecule reaction between C^+ and ammonia leads to HNC rather than HCN. It is often stated that H_2CN^+ has the structure HCNH^+ . While this seems to be the most stable isomer¹⁰ there is no reason to doubt that the other isomers H_2CN^+ and H_2NC^+ correspond to alternative, locally stable arrangements. Unimolecular rearrangements of these less stable isomers to HCNH^+ would be exceedingly slow at the temperatures prevailing in molecular clouds.

In place of equations (3) and (4) as the main means of producing HCN, the following scheme seems to be the most plausible



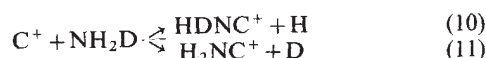
In addition to these processes there is the following rapid isomerisation channel for HNC which, of course, is another process for production of HCN:



Calculations on the relative stability of HCN and HNC (ref. 11) imply that the former is more stable by 0.63 eV and this is supported by some laboratory studies of the equilibrium (ref. 12 and unpublished results) so that the equilibrium ratio HNC/HCN is about 10^{-30} at 100 K. The fact that column densities of HNC comparable to those of HCN have been observed in dark clouds indicates that equilibrium between the two species is not established. This is an important observation because its consequence is that related equilibria involving HNC and HCN in proton exchanges likewise will not be established (they would have to be established at rates much faster than equation (9) which is expected to occur at the Langevin rate and with one of the most abundant ionic species in dark clouds). Also because the equilibrium involving equation (9) has as fast a rate constant and as high concentrations (according to the numerical results of the ion–molecule reaction scheme obtained by Herbst and Klemperer, ref. 7) as any other process considered in discussions of deuterium enrichment through chemical equilibration, it is difficult to sustain such interpretations of molecular deuterium enrichment in dense molecular clouds.

We conclude that the explanation of molecular deuterium enrichments in interstellar HCN and HNC must lie in isotope effects that influence the formation processes rather than equilibria involving HCN or HNC.

In the case of HNC the deuterium is introduced, according to reactions (5) and (6) through NH_2D :

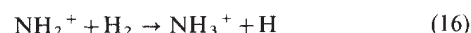
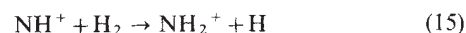


expected in low temperature conditions owing to the substantially greater zero point energy of an N–H bond (typically the zero-point energy difference is $\sim 800 \text{ cm}^{-1} \equiv 1,200 \text{ K}$). Also a similar preference is expected in the second step



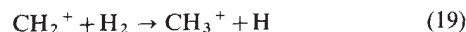
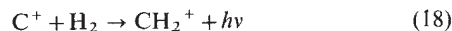
Such preferential decompositions, equations (10) and (12), lead to a DNC/HNC ratio equal to that of $\text{NH}_2\text{D}/\text{NH}_3$, that is, to a threefold deuterium enrichment compared with that in ammonia.

The mechanism^{7,13} of ammonia production in dense molecular clouds is believed to be



where M^+ is a readily ionisable heavy atom such as Ca, Mg, Si. The formation of NH_2D therefore originates from processes analogous to equations (14), (15) and (16) involving HD. The effect of zero point energies will again be to lead to preferential retention of deuterium in the product molecular ion so that a given abundance ratio of HD/H_2 could yield a ratio $\text{NH}_2\text{D}/\text{NH}_3 = 3(\text{HD}/\text{H}_2)$. Overall we might expect an enrichment of DNC/HNC up to three times HD/H_2 corresponding to an overall sixfold enrichment of deuterium as compared with that exhibited by the dominant species in the molecular cloud (H_2) and thus of the cloud as a whole.

For HCN we need to consider deuteration of species leading to CH_3^+ or CH_3 . In the case of the former the processes¹⁴ are probably



which implies that the $\text{CHD}^+/\text{CH}_2^+ = \text{HD}/\text{H}_2$ and $\text{CH}_2\text{D}^+/\text{CH}_3^+ = 2(\text{HD}/\text{H}_2)$. Thus if equations (18), (19), (8) and (4) show the dominant processes for HCN production we could expect an overall deuterium enrichment of HCN of up to four times that of the cloud as a whole. It is not yet clear what is the main channel of production of CH_3 but if it involves



analogous to equation (17) then we expect the same degree of enrichment to arise for the equations (7) and (8). Thus in either case the expected enrichment of HCN is less than that for HNC.

If, however, HCN is predominantly produced from HNC according to equation (9) then the enrichment corresponds to that of the D^+/H^+ ratio because it is this final step that introduces the proton or deuteron that ends up in the HCN or DCN. For dense clouds it is not obvious that the D^+/H^+ ratio will exceed that of the overall deuterium/hydrogen ratio for the cloud and so, to the extent that HCN is produced by HNC, we expect diminished deuterium enrichment for this molecule.

While this implies that a greater enrichment of deuterium will be observed for HNC than for HCN, and this is in keeping with the limited observations so far made, it does not account for the very great enrichments, about 100-fold for HCN, 2,000-fold for HNC so far reported^{3,4}. Since it does not seem feasible for any relevant chemical equilibria to be established on the time scales available for dense interstellar clouds we are forced to turn to kinetic isotope effects. These ion–molecule reactions, however, are all believed to proceed at rates close to the Langevin rate and this leaves little scope for isotope effects.

Perhaps the dissociative recombination reactions in equations (4) and (6) exhibit isotope effects of the requisite magnitude,

As these competing channels involve the fission of an N–H and N–D bond respectively, a very strong preference for the former is

although the theory of secondary isotope effects in unimolecular reactions¹⁵ indicates that the deuterium-containing species would decompose more slowly at the low pressure limit. It seems important to clarify this, either experimentally or theoretically. It would help if the $\text{NH}_2\text{D}/\text{NH}_3$ ratio could be established by observations. Resolution of the problem is urgent because otherwise we must conclude either that the ion-molecule scheme of galactochemistry is inadequate or that the dense molecular clouds are enriched overall in deuterium by a handsome margin compared with the 'standard' cosmic abundance of $\text{D}/\text{H} = 2 \times 10^{-5}$.

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Lost Pacifica continent

THE Alpine mountain chain is generally accepted to be the product of continent-continent collisions. In this belt the zone of recent tectonic activity is wide (up to 2,000 km in Tibet) and crustal thickness in places is 1.5–2 times the average continental crust, presumably due to the inability of light continental material to sink into the asthenosphere. Under the Himalayas, for example, the crust is 70 km thick¹. Furthermore, as indicated by seismicity, the active collision zone here includes not only the highly deformed Himalaya belt but also the entire Tibet plateau. Major wide mountain belts exist, morphologically similar to the Alpine belt, in regions which do not experience continental collision, such as western North America, Alaska, east Siberia and the Andes. The crustal thickness here can also be very great, up to 70 km in the Andes². All are seismically active, wide, highly deformed and include high plateaus of various sizes. Many of these wide orogenic belts also exhibit great geological complexities which are not simply explained by the model of an oceanic lithosphere under-thrusting a continental lithosphere. We suggest, therefore, that the circum Pacific mountain belts may be the result of past continental collisions, similar to those associated with the Alpine belt. We summarise the evidence for the incorporation of past continental masses around the Pacific Ocean. Holmes³ has given a compelling case for large continental land masses during parts of late Palaeozoic to early Tertiary to the west of North America such as Cascadia and Llanoria⁴. The land includes conglomerates derived from crystalline sialic rocks which have since disappeared. Hamilton⁵ and Davis and Armstrong⁶ suggested that the Klamaths were originally some distance offshore to the west and that the Permo Triassic Sonoma Orogeny results from an arc continent collision.

A large scale collision of Alaska with a continental fragment during Palaeozoic and early Mesozoic, finally coalescing in late Jurassic-early Cretaceous times has been suggested^{7–9}. Hamilton⁵ has proposed that Permian terrains

bearing Tethyan fusulinids may have formed in the central Pacific on island arcs which were subsequently swept into the North American continent. Furthermore, these North American terrains share Jurassic and Cretaceous faunas and floras with New Zealand, Caledonia, the Antarctica peninsula and Chile. This is consistent with several palaeomagnetic studies^{10,11} which suggest that large fragments in the western USA, Canada, and in Alaska were located near the equator perhaps at Triassic times. Hillhouse¹² found that tholeiitic flows in the Wrangell mountain area were formed at 15° north or south of the equator during Triassic time.

Jones *et al.*¹³ have shown that a large continental block—Wrangellia—was incorporated in north-western North America in late Mesozoic time. This block(s)¹⁴ extending over 2,000 km from Alaska to Oregon, were not contiguous to central Alaska at Jurassic time.

Most remarkably, the displaced Wrangellia block apparently received enormous quantities of Triassic tholeiitic basalts—to become one of the largest domains of non oceanic basalts¹³. Jones proposed that presumably, rifting initiated this volcanism, but where it occurred and what was rifted remain enigmatic.

In Central and South America there are numerous old basement inclusions within the mobile belts, some of which extend well into the Pacific Ocean itself—suggesting past continental collisions. During the Jurassic, South America was bound to the west by volcanic rocks resting on strongly folded and metamorphosed rocks off Patagonia¹⁵, Ecuador¹⁶, Peru^{17,18} and Bolivia¹⁹. Old Precambrian continental basement fragments, of unknown origins, were recognised also in the Santa Marta mountains in Columbia, the Serrania de Baudo in Panama and Columbia, the Pacific Ocean off Peru, the Nicoya complex in Central America, and the Sierra Madre de Sur in Mexico¹⁸, among others. Palaeomagnetic data²⁰ suggest that Honduras was in the Pacific in Cretaceous times.

The situation is perhaps best summarised by James²²: Jurassic volcanic rocks in southern Peru are wedged in among crystalline metamorphic rocks at least 400 Myr-old. What these remnants of ancient sialic crust are doing some 300 km west of the currently exposed geosynclinal rocks of the continental margin is unknown. These rocks could be part of the palaeozoic microcontinent that lay to the west of the South American coastline.

In north-east Asia palaeomagnetic data²² suggest that the Kolyma block and the Sikhote Alin region have been welded onto the Asian continent, probably in late Jurassic or Cretaceous times. The Verkhoysk and Sikhote mountain belts represent, therefore, probable sites of continental collisions²³. Other possible continental fragments from the Pacific Ocean are the Sea of Ohotzk and surrounding areas in early Tertiary time, almost all of China south of 40°, and Korea^{24,25}. All this may suggest that large fragments have collided with mainland Asia during Triassic through Cretaceous time. Furthermore, Churkin and Eberlein¹⁴ point out that Permian rocks lie outboard of poorly known Palaeozoic and Precambrian rocks in the Alaska range. Ancient rocks reappear west of the Bering Sea along the north-west rim of the Pacific, where similar terrains of Palaeozoic age occur in north-eastern USSR, in Japan, and discontinuously further south along the west Pacific rim. Thus not unlike North America and the Andes, mysterious continental masses in the Pacific have been involved in East Siberia, China and Japan. Many of these bodies bear strong evidence for continental origins as indicated by the nature of the old rocks exposed.

We propose that these chunks were parts of a continental mass which has disaggregated, perhaps the way Gondawana has, and Africa is and may continue to disaggregate. We envisage that the circum Pacific fragments were embedded in the major plates of the Pacific Ocean—the Kula,

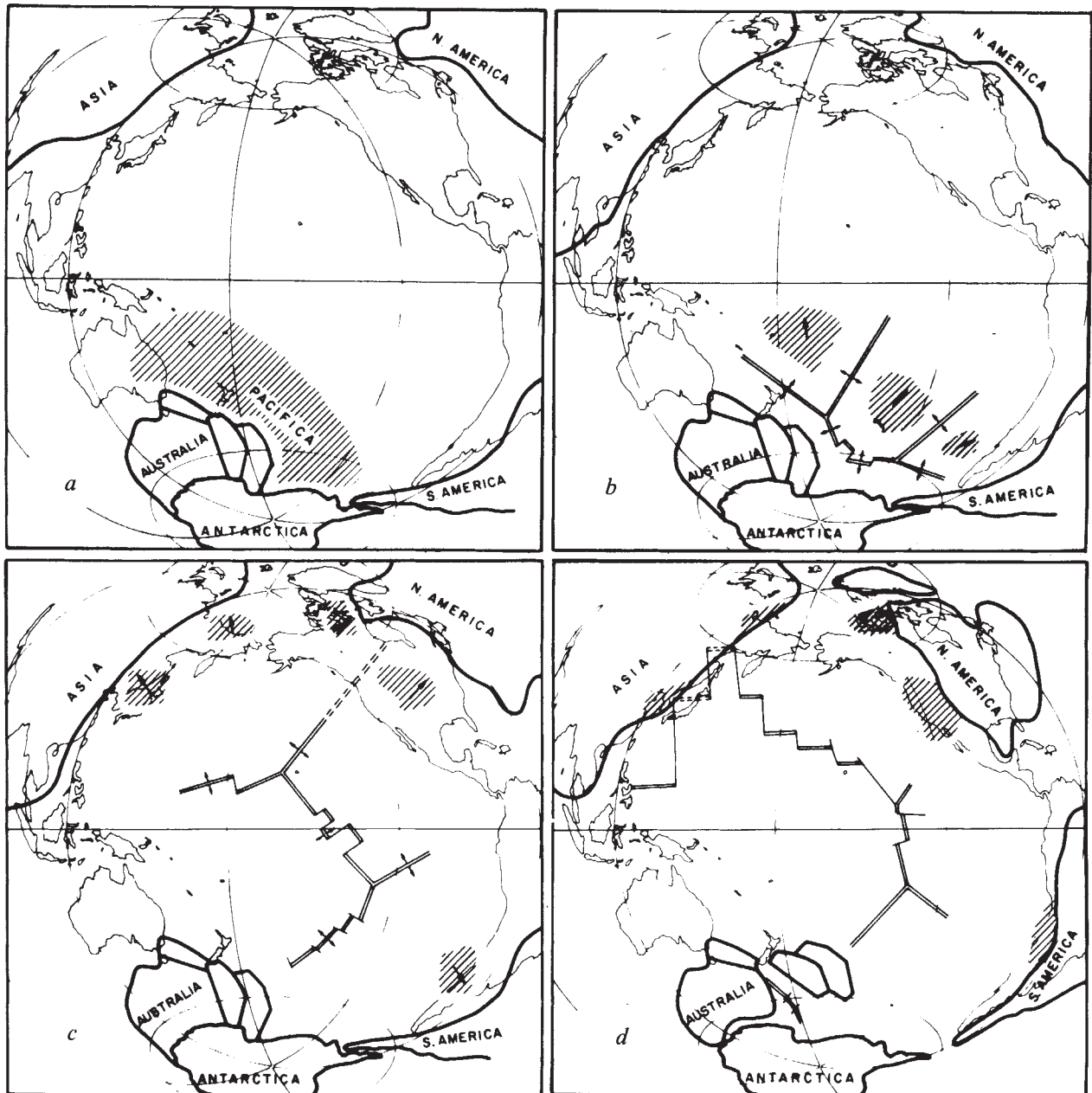


Fig. 1 Schematic model of the breakup of Pacifica and the resulting collision events. Possible ages of the reconstruction stages: *a*, 225 Myr; *b*, 180 Myr; *c*, 135 Myr; *d*, 65 Myr. Fine lines mark the present day continental outline. Heavy lines mark the location of the various continental areas through the geological evolution after Dietz and Holden²⁸. Position of spreading centres simplified from refs 25, 26, 40–42.

Farallon, Phoenix and Pacific plates—whose motion might roughly be reconstructed from palaeomagnetic data back to 190 Myr BP^{25,26}. As shown schematically in Fig. 1 we do this by removing continental blocks from Alaska and western North America, the Andes, Kamchatka and Japan, displacing them in a cartoon like fashion back in time attached to their corresponding plates. The various fragments, as they migrate back towards their respective spreading ridges, also approach each other. By further extrapolating the plate motions backwards beyond Jurassic time, we suggest that they comprise a single mass perhaps by mid-Permian times.

We call this mass Pacifica³²—to emphasise its centrality in the Pacific geological history. Assuming that Pacifica was located over the developing spreading pattern of Larson and Chase²⁵ we imagine four major groups of continental fragments—one each on the Kula, Farallon, Phoenix and Pacific plates. As spreading continued, these fragments

were presumably carried along toward subduction zones, eventually reaching continental margins. Roughly speaking the Kula fragments collided with Alaska and Eastern Siberia, the Farallon fragments with North America and the Phoenix fragments with South America. The submerged platforms in the south-west Pacific (which show typical crustal structures) such as the Ontong-Java and Manihiki platforms may thus be fragments of Pacifica.

Before its breakup Pacifica could have been somewhere in the neighbourhood of Australia, as shown in Fig. 1. We chose to extend Pacifica to Proto Antarctica, to account for the possibility that southern South America may be a splinter off Antarctica²⁷ and that at least parts of the Antarctica coast does consist of rifted margins²⁸ indicating continental rifting.

The existence of Pacifica may thus explain the origin of the circum Pacific Cordillera, and probably shed some light on the origin of the submerged platforms in the

south-west Pacific Ocean^{29,30}. It may provide the continental connection between western North America, south-east Asia, Australia, and South America, needed to explain the evolutionary history of flora, such as the angiosperms^{31,32}, and fauna, such as various fusulinids³ and mammals^{33,34} around the Pacific. In fact, the concept of a Pacifica was first introduced by biogeographers^{32,35-37} solely to explain the relation between the species and families surrounding the Pacific. Our results may, therefore, provide the geophysical and geological detail necessary to understand the continental and biological history in the Pacific. We believe that the combined evidence from geophysics, geology and biology makes a compelling case for a now extinct Pacifica continent, whose fragmented remains are mostly now embedded in the circum Pacific mountain belts.

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Rb-Sr ages of early Archaean supracrustal rocks and Amitsoq gneisses at Isua

PREVIOUS field descriptions¹⁻⁴ and isotopic age determinations^{5,6} left unresolved several important points about the relationships between the Isua supracrustal rocks and the surrounding Amitsoq gneisses. In scattered areas of comparatively low deformation, field relationships show unequivocally that

gneissose granitic veins intrude the supracrustal sequence. Geochemical studies⁷ show that these veins are closely comparable to early tonalitic components in the polyphase Amitsoq gneisses surrounding the supracrustal belt. Here we show that the veins are isotopically indistinguishable from the main outcrops of Amitsoq gneisses. They do not represent a period of granitic activity significantly younger than the Amitsoq gneisses, such as the ~2,900 Myr-old Nûk gneisses of the Godthaab area. Thus the veins may be classified as Amitsoq gneisses. Present field and isotopic evidence combined suggest that at least part of the ~3,700 Myr-old Amitsoq gneisses are younger than the Isua supracrustal belt. We have found no field or isotopic evidence that any part of the Amitsoq gneisses so far mapped pre-date the deposition of the supracrustals.

It was also uncertain whether quartzofeldspathic gneisses so far studied isotopically from the Isua area (West Greenland) were representative of the main body of Amitsoq gneiss in that area. The earlier material studied was collected largely from gneisses close to the supracrustal rocks. Most of the samples are strongly foliated rocks in which primary igneous features had been lost during deformation. In contrast Amitsoq gneisses with original structures away from the immediate contact of the supracrustal succession were represented by only a few samples. It was thought possible that the rocks in the contact zone could represent a separate unit, either a screen of granite material emplaced along a tectonically controlled zone between an old gneiss basement and a younger cover or a group of greywacke-like sediments deposited on gneissic basement.

The material already studied isotopically^{5,6} was supplemented by samples collected by Bridgwater and McGregor in 1973 of which the field relations were better known. Three groups of rocks were selected for further Rb-Sr age study: (1) veins of tonalitic gneiss up to several metres in width which cut across an earlier foliation in Isua amphibolitic supracrustal rocks outcropping to the west of the lake Imarssuaq (Fig. 1). These have tonalitic or granodioritic compositions comparable to the majority of Amitsoq gneisses (samples 158528-530). (2) Massive, granodioritic Amitsoq gneiss collected approximately 2.5 km north of the contact between supracrustals and gneisses east of Imarssuaq (samples 117991-993). (3) Homogeneous, muscovite-bearing, leucocratic schist found as a major unit over 100 m wide within the supracrustal succession to the west of Imarssuaq (samples 158509, 158526, 158526 A). These were considered by Bridgwater and McGregor to be either acid volcanics or more highly deformed equivalents of the discordant tonalitic veins described in (1) above. Thin section and chemical studies⁷, however, have shown that they are closely related to K-rich boulders found within the conglomeratic unit which forms a conspicuous part of the southern and eastern parts of the Isua succession¹⁻⁴. Further mapping by Allaart^{3,4} has shown that this unit of leucocratic schist adjoins the westward continuation

Table 1 Rb-Sr analytical data

Sample no.	⁸⁷ Rb/ ⁸⁶ Sr*	⁸⁷ Sr/ ⁸⁶ Sr	Rb (p.p.m.)†	Sr (p.p.m.)†
Group 1				
158528	0.288	0.7165 ± 1	43	490
158529	0.310	0.7171 ± 1	22	206
158530	0.996	0.7556 ± 1	47	149
Group 2				
117991	0.787	0.7413 ± 1	91	336
117992	0.898	0.7471 ± 1	98	315
117993	0.945	0.7521 ± 1	95	294
Group 3				
158509	0.901	0.7495 ± 1	68	220
158526	5.84	1.0133 ± 2	139	71
158526A	3.36	0.8807 ± 1	93	82

*Determined by X-ray fluorescence, average precision ±1%.

†Mass absorption coefficients estimated from background; concentration data ~ ±5%.

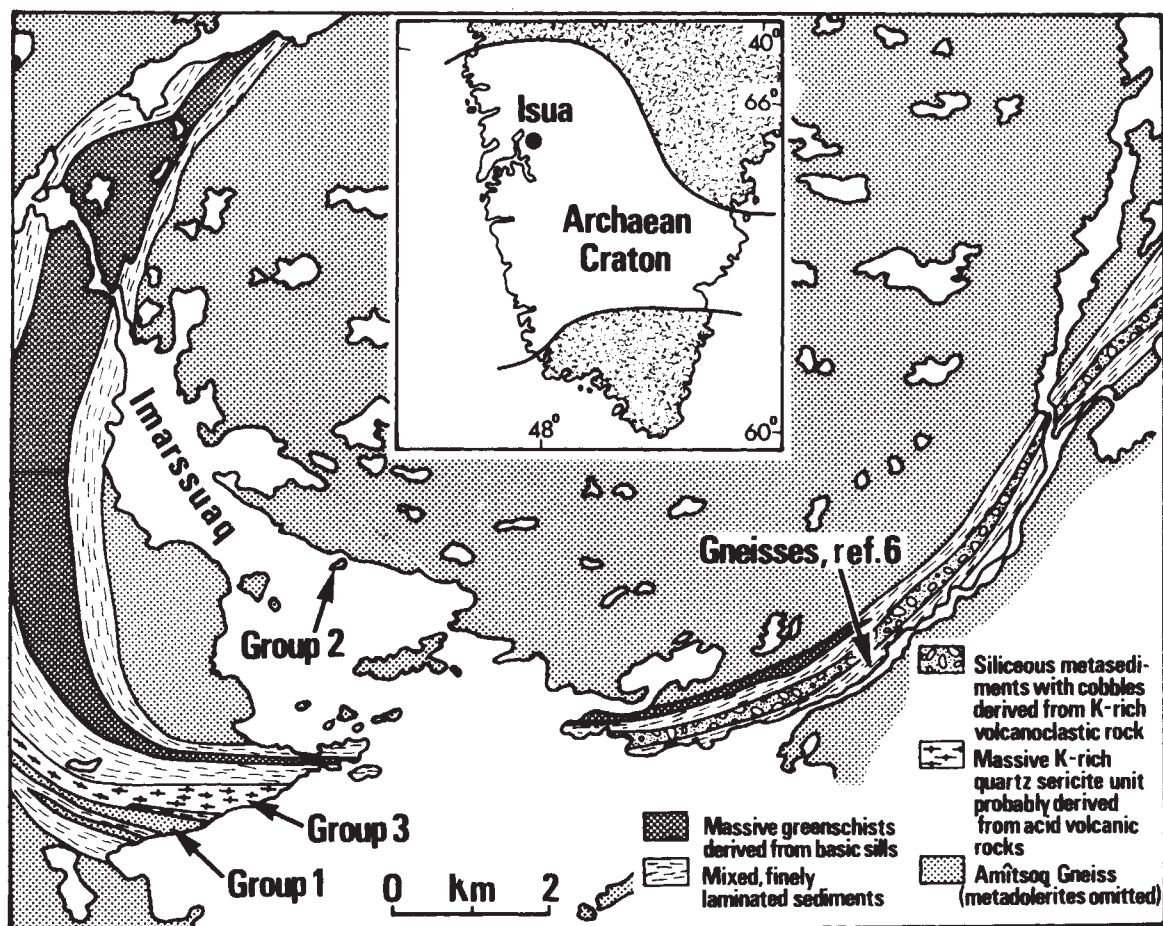
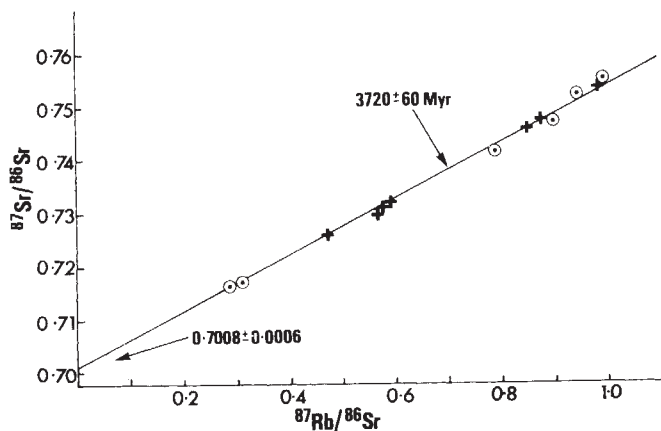


Fig. 1 Simplified geological sketch map of Isua area, after Allaart^{3,4} with specimen localities shown.

of the conglomerate beds. The muscovite schists are now regarded as belonging to the same suite of acid, volcanogenic rocks as the conglomerate. They may be derived from acid lavas that were the source rocks for many of the boulders found in the adjacent volcanogenic conglomerate.

Table 1 shows Rb-Sr whole rock analytical data on three samples from each of the three groups listed above. Full details of chemical and mass-spectrometric techniques have been described elsewhere⁸. Rb/Sr ratios were determined by a precise X-ray fluorescence technique⁹. The decay constant of ^{87}Rb was taken as $1.39 \times 10^{-11} \text{ yr}^{-1}$.

Fig. 2 Rb/Sr whole rock isochron plot for Amitsoq gneisses from Isua. $\odot$, Gneissic veins cutting supracrustals (Group 1, Table 1) and Gneisses far away from contact with supracrustals (Group 2, Table 1). $\pm$, Gneisses from near contact with supracrustals (for full details and analytical data, see ref. 6).



The groups are rather small to be treated individually but may be regressed in combination with each other and with published data. Groups 1 and 2, respectively representing gneissic veins cutting supracrustal rocks and Amitsoq gneiss well away from the contact, give a combined isochron age of $3,750 \pm 200 \text{ Myr}$ (all errors on ages quoted at 2σ), and initial $^{87}\text{Sr}/^{86}\text{Sr}$ of 0.7008 ± 14 (also quoted at 2σ). The isochron fit is not perfect and analytical errors would have to be magnified by a factor of 3 to achieve perfect fit. When these data are regressed together with a published 7-point whole-rock isochron⁶ of $3,780 \pm 130 \text{ Myr}$ and initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.6998 ± 12 for Amitsoq gneiss from close to the margin with the supracrustal rocks, the combined data yield an isochron giving an age of $3,720 \pm 60 \text{ Myr}$ and initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratio of 0.7008 ± 6 (Fig. 2). This demonstrates that the gneissic veins of Group 1 and the Amitsoq gneisses were formed within a period covered by the analytical error on each rock suite. We, therefore, consider them to be contemporaneous and that the Isua supracrustals pre-date the Amitsoq gneiss.

The three samples of Group 3 yield an isochron with an age of $3,760 \pm 70 \text{ Myr}$ and initial $^{87}\text{Sr}/^{86}\text{Sr}$ of 0.7014 ± 15 . When regressed together with five out of six published analyses⁶ on the probably closely related (see above) boulders and matrix from the Isua conglomeratic unit^{3,4,7}, the combined isochron gives an age of $3,740 \pm 60 \text{ Myr}$ and initial $^{87}\text{Sr}/^{86}\text{Sr}$ of 0.7015 ± 17 (Fig. 3). (The omitted sample from ref. 6 is 158538.) The six published analyses gave an errorchron of $3,860 \pm 240 \text{ Myr}$, with an intercept of 0.675 ± 40 . The far more precise results reported here are due to the much greater range of Rb/Sr ratios for the combined data, as well as the omission of one of the published values. The revised age agrees well with a Pb-Pb isochron age of $3,760 \pm 70 \text{ Myr}$ for the banded iron formation member of the Isua supracrustal sequence⁵.

The following principal conclusions may be drawn from these data. First, gneissic veins which intrude the Isua supracrustal sequence yield Rb–Sr whole rock ages indistinguishable from the surrounding Amitsoq gneisses. Together with fragments of greenstone belt-type lithologies (Akilia association) enclosed in Amitsoq gneisses elsewhere in the Godthaab district¹⁰ the Isua supracrustals are the oldest rocks recognised in the Archaean of West Greenland.

Second, the Isua supracrustals and surrounding Amitsoq gneisses are all between ~3,700 and 3,800-Myr-old, although the actual age difference between the two rock units is probably too small to resolve by Rb–Sr and Pb–Pb whole-rock age methods. U–Pb methods on zircons may offer more hope in this respect^{11,12}. Thus, a U–Pb zircon date of $3,600 \pm 50$ Myr has been reported on Amitsoq gneisses from the Godthaab area¹¹, whilst a U–Pb zircon date of $3,824 (+12 \text{ or } -9)$ Myr has been reported from metavolcanic boulders in a large conglomeratic unit within the Isua supracrustals¹². Unfortunately, no zircon U–Pb dates have yet been published for the Amitsoq gneisses at Isua.

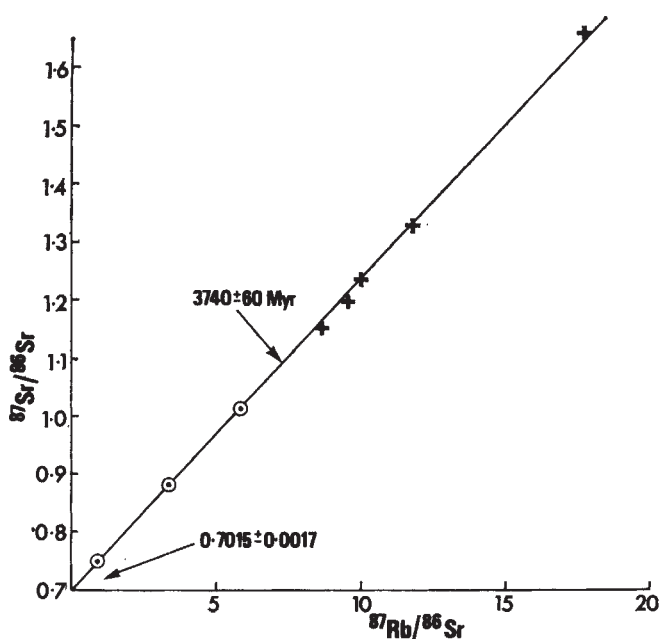


Fig. 3 Rb–Sr whole rock isochron plot for Isua supracrustal rocks. ○, Schists (Group 3, Table 1). ×, boulders and matrix from conglomeratic unit (for full details and analytical data, see ref. 6).

Finally, initial $^{87}\text{Sr}/^{86}\text{Sr}$ ratios are in the range ~0.700–0.702 and suggest, in agreement with previous views^{13,14}, that the precursors of these early Archaean rock units had not existed with average crustal Rb/Sr ratio for more than about 100 Myr before the measured age.

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Laser speckle photography in a fluid medium

A TECHNIQUE for obtaining quantitative velocity data from hydrodynamic flow fields using laser speckle photography (LSP) has been developed and uses the scattered light from the interior of a suitably seeded liquid which is illuminated by a coherent beam from a pulsed ruby laser. The resulting speckle pattern can be photographed on high resolution film. A doubly exposed photograph of the correlated speckle patterns produced by fluid dynamic motion contains all the information necessary to describe the motion throughout a selected plane. When the speckle photographs are optically interrogated distinct fringe patterns are produced whose geometries are related to the velocity field. Here we describe how a Poiseuille flow was used to demonstrate this novel technique. Doubly exposed speckle photographs and typical fringe patterns illustrating the velocity distribution are given, analysed and compared with the classical theory.

The LSP technique has been used since 1969 almost exclusively for various applications to surface metrology^{1–3}. Barker and Fournay⁴ have extended the method to measure displacements in the interior of a transparent solid by recording side-scattered light from an illuminated domain. With lasers of conventional power such recordings require very long exposures. Consequently, the few applications reported so far have examined only imposed static strains. The basic difference between that work and the present application to fluids is that the latter is truly dynamic involving moving liquids rather than static solids.

The extension of side-scattering speckle techniques to fluid dynamic situations has been achieved by using a double pulsed Q-switched ruby laser and a suitably seeded medium. The experimental arrangement used to record speckle photographs of the flow field is shown in Fig. 1. The beam from a ruby laser, which

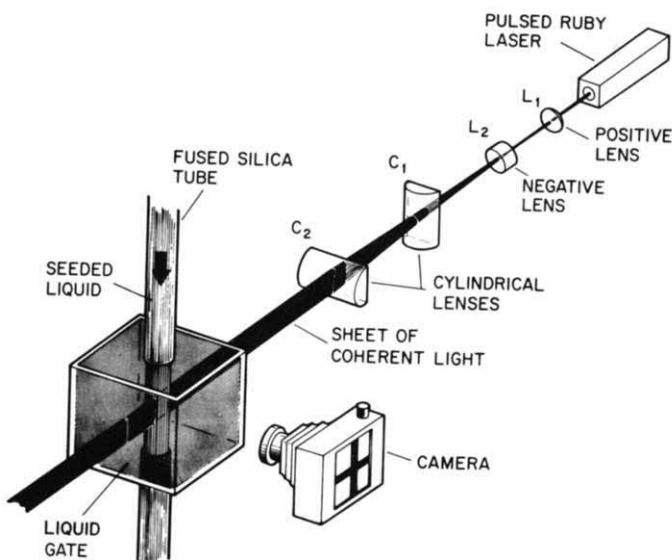


Fig. 1 Experimental arrangement for side-scattering LSP.

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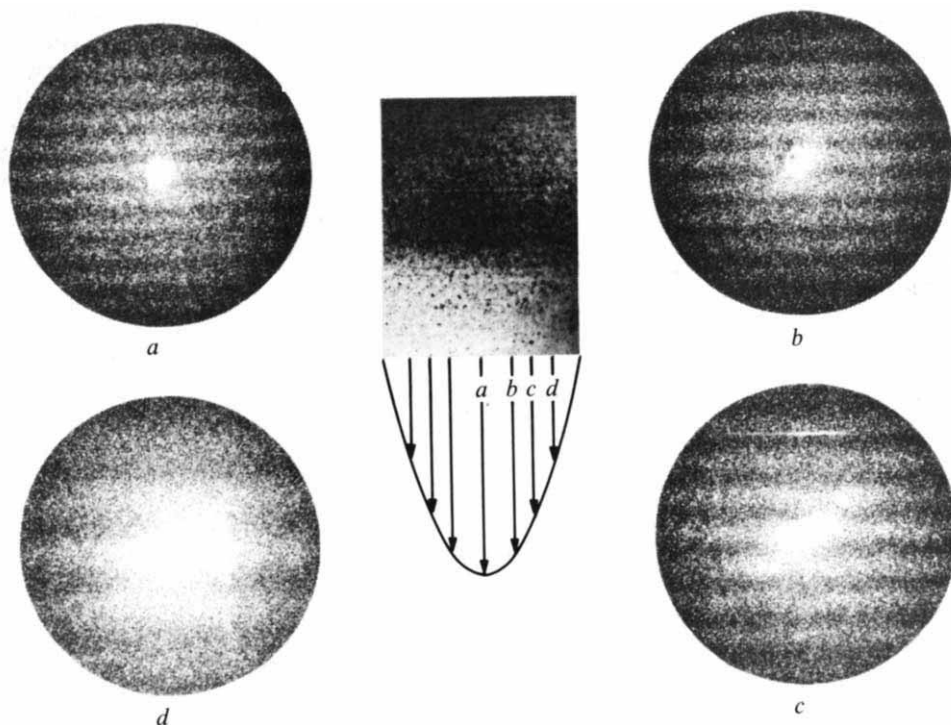


Fig. 2 Typical laser speckle photograph and diffraction fringe patterns from radial locations as shown; *a*, $r/R = 0$; *b*, $r/R = 0.32$; *c*, $r/R = 0.53$; *d*, $r/R = 0.74$.

delivers 2.4 J in each 20-ns pulse, is condensed by lens L_1 . A negative lens L_2 is placed ahead of the focal point of L_1 . From the virtual focus of L_2 the beam is expanded and collimated into a vertical planar sheet about 40 mm high and 1–1.5 mm thick by the cylindrical lenses C_1 and C_2 . A slit may be inserted at the observation cell to assure a beam of uniform 1 mm thickness, but is not essential. The above arrangement of optical components specifies the interior plane of study and optimises the amount of light available to be side-scattered from the flow field.

To demonstrate the technique, a Poiseuille flow was established in a quartz tube using glycerine seeded with a minute quantity of

white latex paint (~ 0.01 ml per l). Refraction effects due to the curvature of the tube were minimised by enclosing the illuminated portion in a liquid gate filled with an index matching fluid. The correlated speckle patterns were recorded on Agfa-Gevaert 10E75 high resolution film. Double-exposed speckle photographs, with time separations of 10–15 ms were recorded at a magnification m of 2.1 and lens apertures between $f/2.8$ and $f/4$. At the ruby wavelength $\lambda = 694.3$ nm, the Rayleigh resolution criterion

$$s_0 = \frac{1.2 \lambda f (1+m)}{m} \quad (1)$$

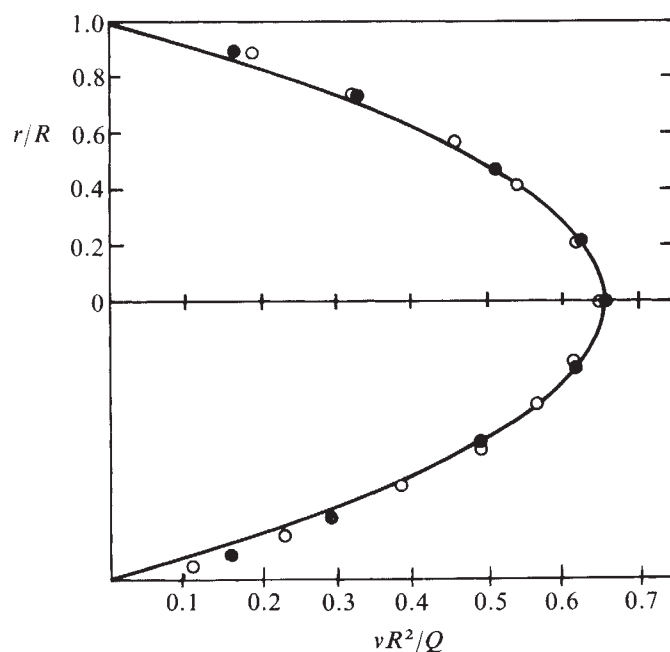
estimates the speckle size in the object plane to be $3.4 \mu\text{m}$ and $4.9 \mu\text{m}$ for the apertures quoted above. These speckles form a very fine random grid which moves with the medium in the illuminated plane. Therefore, a double-exposed photograph of the side-scattered light contains two correlated grids which can be analysed as a non-uniform diffraction grating. This technique can be used successfully only where the displacement between exposures is greater than the speckle diameter, but not so great as to destroy correlation. In this study, which included more than the two examples discussed here, the displacements that were most readily resolved ranged between 2 and 20 speckle diameters.

Figure 2 shows a typical double-exposed speckle photograph, and several diffraction fringe patterns which were obtained by interrogating it at various radii across a diameter of the tube. The interrogation was accomplished by illuminating the speckle photograph with a narrow beam of coherent light which diffracts into a pattern of Young's fringes. The spacing and orientation of the fringes are related to the displacement field at the point of interrogation. Specifically, the displacement of the medium, d , is related to the angular spacing of the fringes, α , and the magnification, m , by

$$\sin \alpha = \lambda/dm \quad (2)$$

where λ is the wavelength of the interrogating beam. In addition, the displacement vector in the illuminated plane is always normal to the direction of the diffraction fringes at each point. Because Poiseuille flow is steady and unidirectional, the fringes are

Fig. 3 Comparison between the LSP data and the classical Poiseuille theory. $\circ$, $Q = 0.53 \text{ ml s}^{-1}$, $\Delta t = 10 \text{ ms}$; $\bullet$, $Q = 0.264 \text{ ml s}^{-1}$, $\Delta t = 10 \text{ ms}$.



everywhere perpendicular to the tube axis and the spacing varies continuously and identically across any diameter. In general, in flow-fields which are not unidirectional the fringe patterns will be less regular.

Analysis of a series of diffraction patterns across a diameter gives the fluid displacement as a function of radius, r . Thus the velocity distribution can be obtained directly since the pulse separation is known. Figure 3 gives the velocity, v , for two time separations and flow rates, Q , compared with the classical distribution

$$v(r) = \frac{2Q}{\pi R^2} \left(1 - \frac{r^2}{R^2} \right) \quad (3)$$

where R is the radius at the wall. The agreement between the LSP data and the classical theory is very good over most of the tube diameter. Near the walls, however, positional errors due to refraction are apparent because of the 0.7% refractive index mismatch between the fused silica and the glycerine.

The side-scattered LSP technique therefore offers an attractive alternative to the existing methods of studying fluid dynamic flow fields. In contrast with conventional interferometric techniques, side-scattered LSP measures the velocity field explicitly, and can examine a uniquely defined interior plane rather than integrating the results through the entire volume thickness.

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Mean residence times in continuous flow systems

INDIVIDUAL elements of a material which enters a system at a constant rate, resides for some time, and then leaves, can take different paths through the system, travel at different speeds, and be delayed for many reasons. For example, by diffusing into stagnant zones, or by transferring, perhaps across a membrane, into a separate phase, they may take part in any activity that does not destroy them which does not preclude chemical reaction where atoms of flow material (which may be combined in any way with other components in the flow streams) are being considered. The process must, however, be a steady-state one, which implies that the quantities of material or 'holdups' in all parts of the system do not vary with time, and that the rate at which material leaves the system is equal to its rate of entry. As an example, we could consider a stretch of river swept by water, or an effluent component in the water; or a continuous chemical reactor into and out of which a conserved material (atoms or ions perhaps) flows at a constant rate; or a respiratory system, fed with oxygen which is transported to the various parts of the body before leaving in part as carbon dioxide. At first sight it seems unlikely that quantitative deductions of the behaviour of so abstract a structure are possible; however, one such deduction has been made¹, and this letter reports another.

The residence time distribution, $f(t)$, of the material component considered, completely defines the general system: thus, the fraction of key material leaving the system, that has resided in the system for a period of time in the interval $(t, t+dt)$, is $f(t)dt$. Buffham¹ has generalised a well established result for simple flow systems, showing that the mean residence

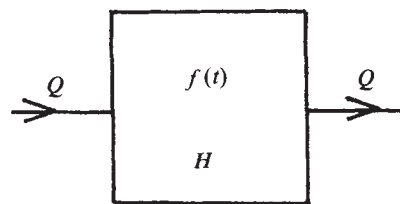


Fig. 1 Block diagram for the general system: the steady flow rate of the key material component is Q and its residence time distribution is $f(t)$; its total holdup in the system is H .

time of material, μ , is independent of the internal characteristics and always equal to the ratio of total holdup of the material to its overall flow rate through the system. Figure 1 and equation (1) summarise this.

$$\mu = \frac{\int_0^{\infty} tf(t)dt}{Q} = H/Q \quad (1)$$

Obvious uses of this are in the measurement of the holdup of the component, or its overall flow rate, where the residence time distribution can be determined by tracer techniques², but its usefulness goes well beyond these direct applications. For example, it has been central to the development of a model-independent theory of chromatography¹ which supercedes previous treatments, based on specific models of the chromatographic process, by obviating the restrictive assumptions on which those are founded.

We shall now use the basic relationship shown in equation (1) to make deductions about arbitrarily defined internal regions of the general system; a more complete proof of the final result, equation (2), will be published elsewhere.

Consider the general system divided into n regions having holdups of the key flow material $h_1, h_2, \dots, h_n$ where

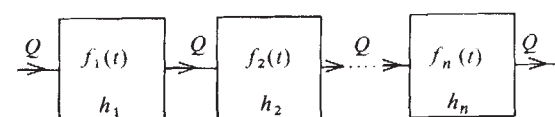
$$\sum_i h_i = H$$

The residence time distributions of the material in those regions is denoted by $f_1(t), f_2(t), \dots, f_n(t)$ respectively. In general, an element may enter a region any number of times; however, periods of residence that make up these distributions refer to total times spent by elements in the respective regions before they leave the system. The period of residence of a material element in the system will be the sum of its residence times in the n regions. Assuming these separate periods of residence to be independent of each other (and it is difficult to think of a steady-state system where this condition is not satisfied), then the distribution of the sum of residence times in the various regions, $f(t)$, may be obtained by convolution³. Thus the n -fold convolution

$$f(t) = \int_0^t \int_0^{\lambda_1} \dots \int_0^{\lambda_{n-2}} f_n(t - \lambda_1) f_{n-1}(\lambda_1 - \lambda_2) \dots f_2(\lambda_{n-2} - \lambda_{n-1}) f_1(\lambda_{n-1}) d\lambda_{n-1} d\lambda_{n-2} \dots d\lambda_1$$

gives the total residence time distribution. This relationship is represented by Fig. 2 which is thus equivalent to Fig. 1.

Fig. 2 Block diagram of the general system that is equivalent, for residence time distribution purposes, to Fig. 1.



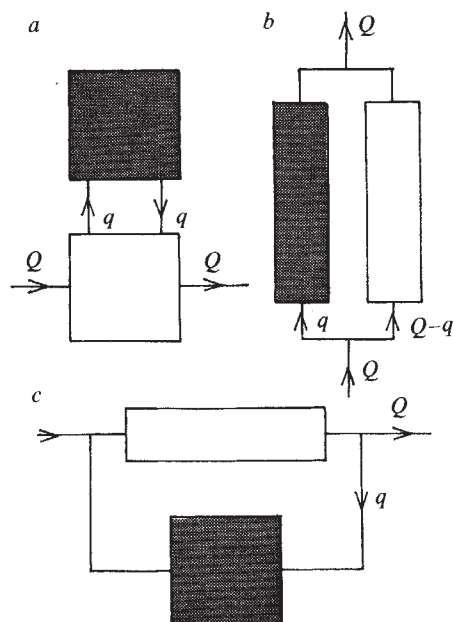


Fig. 3 Examples of simple continuous flow systems for which the general relationship of equation (2) may be readily verified for the shaded regions. Squares and rectangles represent perfectly mixed and plug-flow regions respectively.

Equation (1) may now be applied to any of the blocks of Fig. 2 with the result

$$\mu_i = \int_0^{\infty} t f_i(t) dt = h_i/Q \quad (2)$$

$$i = 1, 2, \dots, n$$

which states that the mean residence time of material in any region of the system is equal to its holdup in that region divided by its overall flow rate through the system as a whole.

This simple result is rather surprising. Figure 3 shows some trivial examples for which it may be readily verified for the shaded regions: although in each case, the residence time distribution of flow material in this region depends on the internal flow rate, q , the mean residence time does not and is given by equation (2).

These examples cease to be trivial if arbitrary mixing patterns are allowed in the regions as well as additional connections between them. Hence, Fig. 3a and b may well model the stretch of river referred to earlier with the shaded areas representing a relatively stagnant backwater; regardless of the descriptive complexities of such a system, equation (2) will hold for both regions and could be applied, say, to making a first estimate of the consequences of an upstream effluent discharge on the ecology of the backwater. Another application could be in predicting the exposure of various regions of a system to a radioactive tracer introduced, for diagnostic purposes, into a flow stream to which they may be only indirectly connected: numerous systems, including physiological ones, are frequently tested in this way.

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Centroceras, the 'missile'-launching marine red alga

VEGETATIVE reproduction by specialised structures is very rare in the red algae. Dixon¹ has listed two cases among freshwater species and two unconfirmed reports from the nineteenth century among marine species. I report here that the marine red alga *Centroceras* produces missile-shaped propagules which are carried away by ocean currents.

Although recorded only six times since its discovery in the Red Sea in 1849²⁻⁸, *Centroceras* seems to be quite common in the epiphytic community of the leaf surfaces of seagrasses, and to a lesser extent, larger attached algae. It is, however, inconspicuous due to intensive grazing by the many herbivorous fish of the region.

'Clean-looking' *Halophila stipulacea* (Forssk.) Aschers, plants were cultured in the laboratory. Undisturbed by fish, the epiphytic algal rudiments attached to the leaves developed within 4 d to give a luxuriant growth clearly visible to the naked eye. Among the algae isolated from this growth was *Centroceras*. (The Red Sea populations have usually been referred to as *C. clavulatum* (C. Ag.) Mont.^{3,6-8} or *Ceramium clavulatum*^{4,5}.) Our *Centroceras* plants differed from other *C. clavulatum* populations in showing: (1) two branching patterns at the apex regions (Fig. 1); (2) limited growth of lateral branches while attached to the parent plants; (3) unpigmented cortical cells of the lowermost internode of the lateral branches; (4) rhizoids just above the first node of the lateral branch, and (5) missile-shaped lateral branches that dropped off and were carried away as propagules.

When they reached the bottom of the culture dish, the propagules resumed growth, attached themselves to the

Fig. 1 Monopodial unilateral branching of 'missile'-bearing branch of *Centroceras* from the Red Sea. Scale bar, 250 μ m.



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substrate by rapid elongation of their rhizoids and elongated rapidly. Within a few days, sometimes as soon as 4 d, the developing plants were large enough (7–10 mm long) to produce 'missiles' of their own. A great many such 'missiles' were produced by one parent plant.

Attempts to find 'missile'-bearing plants of *Centroceras* in *Halophila* beds failed as no *Centroceras* growth could be detected on the leaves. But it was found on leaves of another seagrass, *Thalassodendron ciliatum* (Forssk.) den Hartog. Unlike *Halophila*, *T. ciliatum* has leaves which are largely horizontally orientated, often forming a close canopy which prevents fish from reaching the lower leaves and facilitates undisturbed development of epiphytic algal growth.

In the sea the numerous tiny 'missiles' produced by *Centroceras* plants which escape the teeth of fish, would be carried away easily by currents and spread over large areas, to settle on leaves of seagrasses and other suitable surfaces. They serve both as long-range dispersal units and as a means to ensure quick development of the future plant which in a very short time produces new dispersal units, before falling prey to grazing fish. There are many species of herbivorous fish in tropical seas, and it may be assumed that the production of the missile-shaped propagules by *Centroceras* in the Red Sea and probably in other areas of the Indopacific region, evolved as a result of heavy grazing pressure.

In that case, other delicate algae of similar habitats there and in other tropical regions can be expected to have reacted to grazing pressure by producing specialised vegetative reproductive structures yet to be discovered.

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Phytochrome-regulated organogenesis in lettuce tissue culture

LIGHT intensity and daily photoperiod are known to affect organ development in tissue culture¹, but little information is available on the spectral region influencing organogenesis. Studies with tobacco callus have shown that shoot initiation is enhanced by blue light^{2,3} and that red light has no influence. Red light, on the other hand, promotes callus growth and adventitious bud formation in some species^{4,5} and induces the elongation of endogenous buds in cultured root segments of *Convolvulus arvensis* L.⁶. The latter phenomenon is regulated by phytochrome⁶. In the culture conditions described here, lettuce cotyledon cultures develop well-formed shoots under daily (16-h photoperiod) irradiation from broad-band emitting fluorescent lamps. Dark-grown control cultures, on the other hand, form significantly fewer shoots. We describe here the enhancement of shoot formation in lettuce cotyledon cultures by red light and demonstrate that organogenesis is under phytochrome control⁷.

Explants (3×2 mm) from the distal regions of the cotyledons were obtained from dark-grown, 5-d-old germinated seeds of *Lactuca sativa* var. 'Black Seeded Simpson'. Excision and planting were done under low intensity (~4.5 μW cm⁻²) 504-nm light to which the cultures are not sensitive. The explants were grown on MS salts⁸ supplemented with indole-3-acetic acid 1 mg l⁻¹, kinetin 0.5 mg l⁻¹,

Table 1 Red/far-red reversible effects on shoot formation and callus growth in lettuce cotyledon cultures

Treatment	Sample size	No. of shoots per culture	Callus weight (g) per culture
Expt 1			
Control (no light)	55	6.34±0.56	2.25±0.21
5 min R per d	30	16.96±0.78*	3.25±0.23‡
5 min R+5 Min FR per d	27	6.48±0.92	1.70±0.32
Expt 2			
Control (no light)	85	3.02±0.18	2.76±0.19
5 min R per d	29	6.21±0.47*	4.15±0.24§
5 min R+5 Min FR per d	28	4.04±0.39†	2.20±0.29
Expt 3			
Control (no light)	29	3.69±0.35	3.01±0.23
5 min FR per d	25	3.80±0.33	2.26±0.20

Lettuce cotyledon cultures were exposed to the indicated conditions for 35 d before data collection. R, 240 μW cm⁻² 660 nm light; FR, 680 μW cm⁻² 740 nm light. Errors represent ±1 s.e.m. Statistical significances given in comparison with control values.

*0.001 ≥ *P* (Smirnov test).

†0.05 ≥ *P* > 0.01 (Smirnov test).

‡0.01 ≥ *P* > 0.001 (*t*-test).

§0.001 ≥ *P* (*t*-test).

||0.05 ≥ *P* > 0.01 (*t*-test).

adenine sulphate 40 mg l⁻¹, thiamine-HCl 0.1 mg l⁻¹, nicotinic acid 0.5 mg l⁻¹, pyridoxine-HCl 0.1 mg l⁻¹, glycine 2 mg l⁻¹, myo-inositol 100 mg l⁻¹ and sucrose 30 g l⁻¹ (ref. 9). Before addition of Bacto-Agar (Difco) 10 g l⁻¹, the medium was adjusted to pH 5.8. The 660-nm red light source was described previously³. The far-red source was a narrow-band emitting fluorescent lamp with peak emission at 744 nm (Phosphor E-5346, GTE) from which light below 700 nm was absorbed by FRF-700 plexiglass filter (Westlakes Plastics).

Table 1 shows the results of 5-min daily exposures of lettuce cotyledon cultures to 660 nm, 740 nm, or 660 nm followed immediately by 740 nm light. After 35 d, red light caused a 2- to 2.5-fold increase in the number of shoots and a 1.5-fold increase in callus weight compared with control cultures not exposed to light. Far-red light had no effect on the number of shoots produced but decreased the callus weight slightly. Red followed by far-red light reversed the red light effect on both shoot formation and callus growth. These results demonstrate that both increased shoot formation and callus growth are regulated by the low-energy phytochrome photoreceptor system.

The above data do not indicate at what stage of shoot development red light is effective. For example, light may simply cause elongation of shoots from pre-existing buds as is the case with *Convolvulus arvensis*⁶. To examine this point, both bud and shoot formation were observed at an earlier stage of development. Table 2 shows that red light

Table 2 Red light-induced bud and shoot formation in lettuce cotyledon cultures after 14 d of exposure

Treatment	Sample size	No. of buds	No. of shoots
Expt 1			
Control	30	11.23±0.74	1.47±0.22
5 min R per d	26	20.34±1.14	3.00±0.34
Expt 2			
Control	7	10.00±0.82	1.29±0.52
5 min R per d	23	20.83±1.29	3.09±0.42

Conditions were as in Table 1.

doubles the number of buds as well as shoots present in the cultures after only 14 d of exposure. Thus, light regulation of shoot production occurs at the bud stage of development and perhaps at the bud initiation stage.

To our knowledge this report is the first demonstration of phytochrome-regulated shoot morphogenesis in a tissue culture system. Lettuce cotyledon cultures, then, may serve as a useful, simple, model system for investigating the molecular mechanisms involved in phytochrome regulation of organogenesis.

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Graded localisation of naming from electrical stimulation mapping of left cerebral cortex

APPLYING a weak electric current to the cerebral cortex of awake neurosurgical patients is an established method for mapping language zones on the basis of stimulation-evoked errors. Two new findings are reported here—the topographical extent of language cortex in an individual subject can be wider than that proposed in the classic maps, and, within this zone different sites are variably committed to language as measured by the naming function.

Naming errors evoked by electrical stimulation of the brain in the presence of a retained ability to speak are useful indicators of the language function of focal areas of cortex. Weak electric currents at levels below that which produce after-discharges, deactivate a limited area of cortex abruptly and reversibly, not allowing the brain sufficient time to either compensate or re-organise. Furthermore, research on aphasia has established that lesions to any part of the language system will cause anomia, or naming errors.

In the most extensive studies of stimulation mapping of the cerebral cortex, Penfield and Roberts¹ reported aphasic-like errors from the classical left hemisphere language zones; these errors included distortion and repetition of syllables and words, confusion of numbers while counting, inability to name with retained ability to speak, misnaming and perseveration. What is not generally recognised, however, is that Penfield and Roberts only elicited a total of 132 aphasic errors in 110 patients; of these errors, 19 were misnamings and 51 were omissions with retained ability to speak, the two error categories most representative of anomia.

More recently, Fedio and Van Buren² recorded naming errors from left hemisphere stimulation in 19 patients; in their series, a total of 87 naming errors, primarily omissions, were observed, again clustering in the classic language zones. The small number of sites sampled in any one patient again precluded analysis of the extent and the variability of language representation in the lateral cortex of the individual.

In this report we address two related questions. First, the Penfield and Roberts composite map, derived from pooling the stimulation-error points across subjects, covers a large area of the left hemisphere; is any single individual's language cortex this extensive? Second, are specific areas of cortex within the zone equally involved in language function in a particular individual?

Data bearing on these questions were obtained from three adults with psychomotor seizures from left temporal or left temporo-parietal foci of early childhood onset. The persistence of the seizures despite anticonvulsant therapy indicated surgical treat-

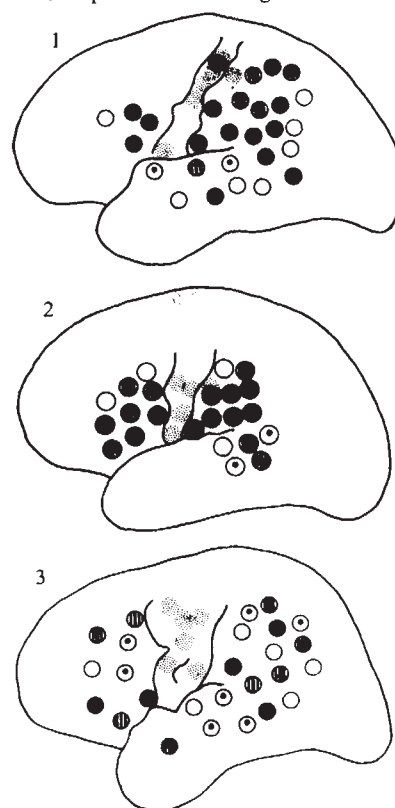
ment. Pre-operative intracarotid sodium Amytal testing, which measured naming, reading and short-term verbal memory, identified the left hemisphere as the dominant one for language in all three patients.

The left fronto-temporo-parietal cortex was exposed at operation under local anaesthesia. Corticography was used to locate the epileptic focus; after that motor and sensory responses to stimulation mapped the Rolandic cortex, which is indicated by stippled circles on the left hemisphere tracing in Fig. 1. Language testing followed, using a set of achromatic slides, each one a line drawing of a different common object such as a bell, a hand, a car, a ring, with the words 'That is a', or 'This is a', printed above. The slides were projected at a constant rate, 4 or 5 s depending on each patient's ease of responding in the test-operative situation. The patient was required to say the words, for example 'This is a', and then to name the object. Testing was carried out in the patient's native language. English for number 1 and no. 3, Dutch for no. 2.

Stimulation was delivered through bipolar silver ball electrodes, 5 mm apart. The stimulations were bi-phasic square wave pulses, 2.5 ms total width at 60 Hz, delivered from a Nuclear Chicago constant current stimulator. The current levels were determined for each patient to be below the threshold for after-discharges in epileptic cortex and below the level of sensation in sensory-motor cortex. The current level for patient 1 was 7 mA in all sites, for patient 2, 8 mA in all sites. For patient 3 it was necessary to use a lower current in the epileptic cortex, 3 mA in the anterior temporal lobe and 4 mA in the posterior temporal lobe; 6 mA were used in the frontal and parietal lobes. Current was delivered in stimulation trains of the same duration as the naming slide presentations, initiated and terminated when the slide projector changed.

A grid pattern of sterile numbered tickets was laid out on the

Fig. 1 Results from naming error tests depicted on left hemisphere angiogram tracings. Solid circles indicate points at which stimulation always produced a naming error in that patient. Shaded circles, points at which stimulation variably produced naming errors at least half of the time. A 50% error rate was chosen as the cut-off point because a lower error rate (given the small number of samples per site) could not unequivocally be distinguished from the non-stimulation control trial error rates. The control trial error rate for patient 1 was 12.8%, for patient 2 was 15.5% and for patient 3 was 14.1%. Sites which had stimulation error rates of less than 50% are indicated by an open circle containing an asterisk. Blank circles indicate sites at which stimulation never produced a naming error in that patient.



exposed cortex of each patient. For patient 1, 28 sites were selected, for patient 2, 23 sites were selected and for patient 3, 26 sites were selected. These sites are indicated in Fig. 1 by open and filled circles. The mean number of stimulation samples per site was 2.3 for patient 1, 2.2 for patient 2 and 3.1 for patient 3. No site was stimulated consecutively; stimulation was not applied during consecutive naming slides. Stimulation was applied to a site only if the immediately preceding object had been named correctly. Non-stimulation control trials were determined by the same criterion, the immediately preceding object had been correctly named.

We elected to score the errors conservatively, counting only omissions and substitutions, and disregarding both hesitations and slurring. If the correct name was spoken before the slide changed, it was counted as a correct trial; if the correct name was spoken after the slide changed, and therefore after stimulation had stopped, it was counted as an error. Substitutions, such as calling a 'fork' a 'spider', were counted as errors. Patient 1 made only one substitution error. Patient 2 made six substitution errors (one of which was the use of an English word for the object instead of the Dutch word). Patient 3 made 17 substitution errors, the only patient of the three who made a large number (43%) of this type of error. In all three patients, 197 stimulations were done at 77 sites, eliciting a total of 99 errors.

The results are depicted on the left hemisphere tracings for each patient in Fig. 1. These tracings were made directly from the venous phase of the angiograms; the sites were located in relation to the cortical veins, using colour photographs taken at the time of the language testing. The data provide an answer to the first question: in each case it was possible to find language functioning cortex throughout the extent of the left hemisphere exposure. Although this observation must be qualified by the fact that these are patients with long-standing epileptic foci, it seems reasonable to conclude that an individual's language system may occupy a very large extent of the lateral cortex, including the classical language zones derived from pooled data from many subjects. Indeed, our data show an even wider language zone than that proposed by Penfield and Roberts in that we find numerous language sites in the parietal operculum and mid-frontal and mid-parietal lobes. The answer to the second question seems to be no; the language function does not seem to be equally distributed throughout the left hemisphere. We repeatedly observed instances of a naming impairment on all stimulations at one site, and only partial or no impairments on all stimulations at an adjacent site, as represented in Fig. 1. The data suggest hypotheses of theoretical interest. If the patterns seen in these patients are indicative of language representation in non-epileptic left hemispheres, and there is no reason to assume that the non-epileptic cortex in these patients is abnormal, it is reasonable to infer that there is a marked degree of individual variation from one person to the next. If that inference is valid, it provides an obvious explanation for both the variety of aphasic symptoms and the variability of recovery of function which follow seemingly similar brain lesions.

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Infective *Theileria annulata* in the tick without a blood meal stimulus

THE agent of bovine tropical theileriosis, *Theileria annulata*, occurs in southern Europe, North Africa and most of Asia. The disease causes high mortality and reduced yield of meat and milk in the survivors. In enzootic areas theileriosis prevents the introduction of improved stock which are in most cases very susceptible. The work described here is part of a programme for improving the effectiveness of the vaccines now used for partial control of the disease. Theileriosis is transmitted in nature mainly by adult ticks that acquire their infection in the pre-imaginal stages. Adults become infective after feeding for a minimum of 2 d either on the susceptible bovine host or on a non-susceptible mammal like the rabbit¹. As parasites can already be seen in the salivary glands of unfed adult ticks that are not infective^{2,3}, it has been assumed that a blood meal provides the biological stimulus for the development of infectivity. In attempting to determine the factors other than feeding, that might stimulate the production of infective stage(s) of *T. annulata*, I have found that a temperature of 37 °C and a relative humidity (RH) of 95% is in itself sufficient to stimulate the production of infective parasite forms in infected adult *Hyalomma excavatum* ticks without the need for a blood meal.

Engorged nymphs of *H. excavatum* that were fed on a calf infected with *T. annulata* were maintained at 28 °C and 65% RH for 6 weeks and the adults that developed were kept for 3 months at 13 °C and 65% RH and served as the tick material for this study. When the adult ticks were allowed to feed for up to 2 d on susceptible calves, the animals were not infected, nor were calves which were injected with the homogenate of these ticks. Calves contracted theileriosis only after ticks had fed on them for 4-5 d, or after being injected with the homogenate of these ticks.

One hundred and thirty ticks from the same batch, enclosed in wire net containers covered with nylon net which prevented them from feeding, were placed in ear bags on a susceptible calf. After 2 d 65 of the ticks were transferred to the earbags on another susceptible calf for 24 h, during which time 38 ticks attached. The calf remained free of theileriosis. After 6 d the 53 confined ticks remaining on the initial calf were removed from the earbags, homogenised and injected into a fresh calf which died with fulminating theileriosis.

Unfed adult ticks were transferred from 13 °C and 65% RH to an incubator maintained at 37 °C and 95% RH for 6 d. The homogenate of 56 such ticks caused lethal theileriosis when injected into a susceptible calf. Unfed ticks which were kept at 13 °C, or ticks allowed to feed on calves for up to 2 d, did not transmit theileriosis either by bite or by needle inoculation.

From these results it seems that a temperature of 37 °C for several days, possibly coupled with high relative humidity, stimulated the non-infective particles of *T. annulata* in the salivary glands of the unfed *Hyalomma* ticks to become infective without the previous stimulation of feeding. These findings might explain the results of Mazlum⁴ who was able to transmit *T. annulata* by injecting unfed infected adult *H. dromedarii* into cattle. Although Mazlum gave no explanation for his unexpected results, the fact that his ticks had been reared at 32 °C and 70-80% RH might have been a significant factor in the experiment.

The unsuccessful attempt by Nuttal and Hindle⁵ to transmit *Theileria parva* by keeping unfed *Rhipicephalus appendiculatus* ticks at 37 °C for 3 d and feeding them on cattle for an additional 2 d, might be related to the species of ticks and the *Theileria* that they studied. The possibility that body temperature of the host, rather than tick feeding *per se* might be responsible for the appearance of infectivity in some tick-borne diseases is supported by the finding⁶ that infective forms of *Babesia bovis* can be produced in unfed *Boophilus microplus* larvae by keeping them

at 37 °C. An elevation in temperature not only causes the appearance of infective particles, but also activates the ticks in general.

Failure to prevent theileriosis in hot climates by dipping cattle in acaricides every 3 d or even daily⁷ might possibly be explained by the rapid appearance of infective particles in ticks exposed to high ambient temperatures, even before they have had a blood meal.

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Endosymbiont as supplier of ornithine carbamoyltransferase in a trypanosomatid

In spite of the widespread occurrence of intracellular bacteria-like symbionts in eukaryotic cells little is known about their function¹⁻³. They are generally thought to offer some advantage to the host cell while benefiting from its 'hospitality', but their precise contribution is known in few cases. Flagellates that harbour endosymbionts provide excellent models for the analysis of this relationship. Three trypanosomatids, *Crithidia oncopelti*, *C. deanei* and *Blastocrithidia culicis* harbour endosymbionts whose bacteria-like nature has been confirmed morphologically and biochemically⁴⁻⁹. Unlike symbiont-free species¹⁰, those species do not require haemin for growth because the endosymbionts provide haemin-synthesising enzymes¹¹. We now report that endosymbionts enable certain species of *Crithidia* to synthesise arginine from ornithine.

Although ornithine carbamoyltransferase (OCT) is characteristically absent from *Crithidia*, it is present in species harbouring symbionts (Table 1). OCT catalyses the first step in the synthesis of arginine from ornithine—the synthesis of citrulline from ornithine and carbamoyl phosphate. Because of its absence, endosymbiont-free *Crithidia* have an absolute growth requirement for arginine or

Table 1 Ornithine carbamoyltransferase in *Crithidia*

Organism	Enzyme specific activity
<i>C. fasciculata</i> (ATCC 11745)	0
<i>C. acanthocephali</i> (ATCC 30251)	0
<i>C. hamosa</i> (ATCC 30256)	0
<i>C. lucillae</i> (ATCC 14765)	0
<i>C. deanei</i> (ATCC 30255)	300
<i>C. deanei</i> without symbiont	0
<i>C. oncopelti</i> (ATCC 30264)	210
<i>C. oncopelti</i> without symbiont	0

Cells were grown in an undefined medium¹⁷. Cell homogenates were obtained by sonication. OCT was determined as before¹⁸. The assay mixture consisted of 40 mM Tris-HCl buffer, pH 8.5, 16.6 mM L-ornithine, 16.6 mM carbamoyl phosphate and 0.8 mg of enzyme protein in a final volume of 0.3 ml. After 15 min of incubation at 37 °C, reactions were stopped by addition of 5% trichloroacetic acid, and the citrulline formed was measured in the supernatant by the Archibald's method¹⁹. Results are expressed in nmol of citrulline per mg of protein per min. *Crithidia deanei* without symbiont was obtained from Drs I. Roitman and M. Mundim, University of Brasilia. *C. oncopelti* without symbiont was obtained from Dr K. P. Chang, Rockefeller University, through Dr Roitman.

Table 2 Growth of three species of *Crithidia* in various media

Organism	<i>C. deanei</i>		<i>C. fasciculata</i>		<i>C. acanthocephali</i>	
Medium	GT	Cells per ml*	GT	Cells per ml	GT	Cells per ml
RDM	8	6×10^7	0	0	0	0
RDM+Orn	6.5	4×10^7	0	0	0	0
RDM+Cit	9	7×10^7	10	4×10^7	6.5	3.6×10^7
RDM+Arg	6.5	7×10^7	10	4.5×10^7	6.5	4×10^7

RDM, Roitman's defined medium¹⁷ without arginine. RDM+Orn contains 3 mM L-ornithine; RDM+Cit contains 2.3 mM L-citrulline and RDM+Arg contains 2.3 mM L-arginine. GT is a rough calculation of the generation time in hours derived from inspection of growth curves.

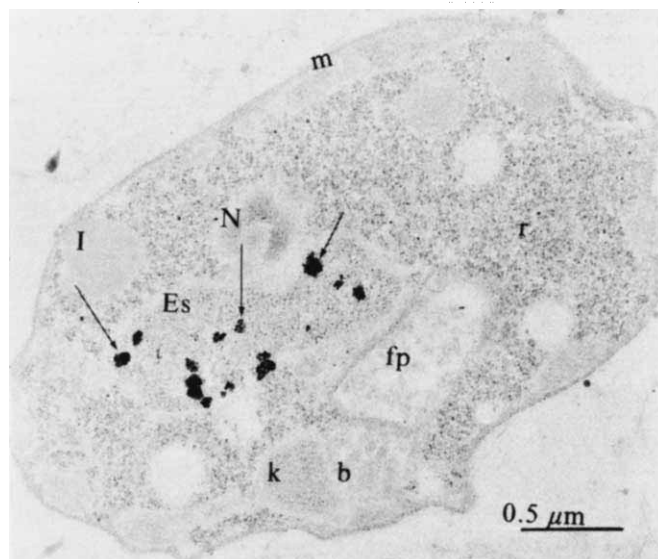
*Number of cells per ml, corresponding to measurements made at the stationary phase of the cultures, before cell decay.

citrulline. *Crithidia deanei* (Table 2) and *C. oncopelti*¹², however, which have endosymbionts can grow in the absence of both amino acids, which indicates that they can synthesise citrulline through the mediation of OCT.

To investigate the role of the endosymbionts, we tested symbiont-free *C. deanei* and *C. oncopelti* for OCT (these strains are obtained by prolonged treatment with chloramphenicol (ref. 8 and personal communication from M. H. Mundim and I. Roitman)). Extracts of the strains showed no OCT activity (Table 1), suggesting that the symbionts provide OCT for *Crithidia*. But because chloramphenicol treatment is likely to affect the mitochondria¹³—reported to be the site of OCT in eukaryotes¹⁴⁻¹⁶—it is possible that the loss of OCT from *C. deanei* and *C. oncopelti* was due to impairment of protein synthesis in their mitochondria rather than to removal of symbionts.

Our efforts to isolate the symbionts and locate OCT within them were inconclusive—we produced subcellular

Fig. 1 Histochemical demonstration of OCT in *Crithidia deanei* which contains an endosymbiont. Arrows indicate OCT reaction product; Es, endosymbiont; N, nucleus; fp, flagellar pocket; k, kinetoplast; b, blepharoplast; l, lipid droplet; m, mitochondrion. Pellets of log-phase cells were fixed for 30 min at 4 °C in glutaraldehyde (2.5% in 0.1 M cacodylate buffer, pH 7.2). After several rinses, pellets were incubated with 5 mM lithium carbamoyl phosphate; 3 mM lead nitrate; 5 mM trisodium citrate; 50 mM Tris-HCl, pH 8.5, and 10 mM L-ornithine for 20 min at 37 °C. In control preparations ornithine was omitted from the incubation mixture. After rinsing with Tris buffer, pellets were included in 3% agar. Small agar blocks were post-fixed in osmium tetroxide (1% in cacodylate buffer, pH 7.2) for 1 h at 4 °C. After dehydration the material was embedded in Epon, sectioned, stained with uranyl acetate and lead citrate and examined in a Siemens Elmiskop 101, 80 kV.



fractions contaminated with mitochondrial fragments. The site of OCT was identified by electron microscopy. Log phase *C. deanei* and *C. oncopeltii* were incubated with ornithine, carbamoyl phosphate and lead nitrate as before¹⁵. In this procedure the orthophosphate released during the OCT reaction is trapped *in situ* as a lead phosphate precipitate which can be visualised in electron micrographs. More than 40% of the cells examined showed a lead precipitate. When present, the precipitate was inside the symbiont and nowhere else in the cell (Fig. 1). No precipitate was found in preparations treated in the same way but without ornithine during incubation, nor in cells of the symbiont-free strains incubated with complete medium. Clearly OCT is localised in the endosymbiont and confers a nutritional advantage on its protozoan host.

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Calcium mobilisation during reproduction in snail *Helix aspersa*

The land snail *Helix aspersa* lays up to 100 eggs at a time, each with 0.6 mg of calcium in the form of calcite crystals. There is insufficient calcium in any part of the reproductive tract to account for this.

We have investigated egg shell calcification in *H. aspersa* and report here a substantial increase in blood calcium during this process while blood sodium and pH remain constant. Rapid calcium mobilisation from non-reproductive areas to the egg shell-forming uterus during this period results in a 70% elevation of the blood calcium concentration, involving an increase in both the bound and unbound fractions.

Of the 65 families of land pulmonate snails comprising about 15,000 species, members of at least 36 families lay eggs with coverings containing calcium carbonate¹. The calcified deposits may take the form of individual crystals of calcite dispersed through a jelly coat as in *Helix* or a hard shell as in *Achatina* and *Strophocheilus*². The egg shell of the snail has four features in common with that of the bird: (1) similarity of mineralogy, ultrastructural appearance and even size in such snails as *Strophocheilus*³; (2) deposition of the shell—concurrently with a small

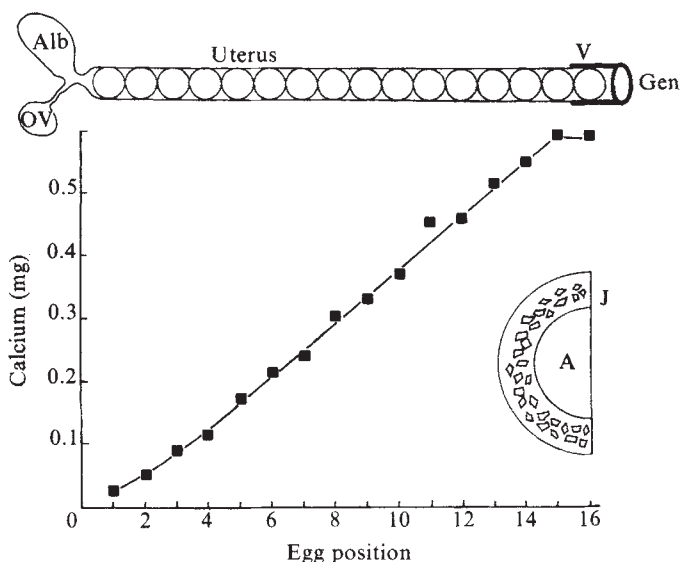


Fig. 1 Calcium content of the eggs as a function of location in the uterus of a gravid *Helix aspersa*. The eggs are in single file with the sides of the uterine wall closely applied, though each egg seems to be free to rotate. As soon as an egg is released anteriorly a new one is formed at the posterior end in an assembly-line fashion. The ovotestis (Ov) and albumen gland (Alb) are shown not to scale, at the posterior end of the uterus. The uterine wall is ciliated and shows strong peristaltic waves moving anteriorly; the vagina (V) is highly muscular and aids in the final extrusion of the eggs through the genital opening (Gen). The calcium content of the uterus after all of the eggs were removed was 0.27 mg. Inset shows a cross-section through a fully calcified egg; the calcium of the egg is in the form of individual calcite crystals dispersed throughout the egg jelly (J), which surrounds the albumen fluid (A) in which the ovum floats. (Correlation coefficient for regression line, $r = 0.99$.)

amount of organic material for the shell matrix—during passage along a tube lined with epithelium; (3) a high rate of calcium transport across the epithelium during shell formation; and (4) utilisation of the shell during embryonic development, in the snail for the shell, in the bird for the bones⁴. On examining several species of pulmonate snails, we found no storage of calcium in the reproductive tract sufficient to account for calcification of even a small percentage of the eggs of a single clutch. Apparently in the snail—as in the bird—calcium stored in other parts of the animal is mobilised and then passes in the blood to the reproductive tract where it is transported across the epithelium to the egg. We have examined egg-shell calcification in the snail *Helix aspersa* and have measured accompanying changes in the blood.

Typically 25-100 eggs are deposited in a single laying period (depending on the size and health of the animal), with an egg released approximately every 15 min. Each deposited egg contains about 0.6 mg calcium requiring a mobilisation of up to 60 mg calcium. The calculated rate of calcium transport across the uterine epithelium of *Helix aspersa* is approximately 3×10^{-6} mol $\text{cm}^{-2} \text{h}^{-1}$, and in another land snail examined, *Anguispira alternata*, the rate is about 1×10^{-5} mol $\text{cm}^{-2} \text{h}^{-1}$. This compares with a calculated rate of about 2×10^{-5} mol $\text{cm}^{-2} \text{h}^{-1}$ for the hen⁵.

Eggs taken from various positions in a gravid uterus of *Helix* were found to have increasing amounts of calcium as they passed along the uterus (Fig. 1), and the amounts were in direct proportion to the distance traversed. This result demonstrates that over most of its length the various regions of the uterus have the same capacity to deposit calcium during passage of the egg. One should note that the uterine epithelium is rather closely applied to the eggs and the eggs are uniformly distributed in single file and

barely separated. There is almost no free fluid in the uterine lumen which can be aspirated so that the eggs are definitely not floating. Free calcite crystals were not present, indicating that crystal nucleation was initiated by the jelly which coats the egg.

During egg laying a dramatic increase in total blood calcium of nearly 70% was found. The elevation was due to an equivalent increase in both the bound and unbound fractions. The blood sodium level did not change significantly and the pH remained at the normal level of about 7.80 (Table 1).

We measured blood calcium levels during the reproductive period as a function of the number of eggs already calcified, and samples were also taken before and after the period of egg laying. Blood calcium suddenly rose from 5.89 mM to 9.93 mM with the onset of egg laying and was maintained for the entire duration of laying (calcification) and then dropped back to the normal level within a few hours after the last egg was released (Fig. 2).

Blood changes in *Helix* differ from those which occur during egg laying in birds in three significant respects: (1) in *Helix*, both bound and ultrafilterable calcium increased, whereas in birds only bound calcium associated with the transport of yolk proteins increases, (2) in *Helix*, blood calcium elevation takes place within several hours of the time of release of the first egg, whereas in birds this occurs several days before egg formation, and (3) in *Helix*, the blood pH is unchanged whereas in birds it is decreased (for a general review of avian egg laying, see ref. 6).

Two aspects of particular interest relating to the rapid mobilisation and utilisation of a relatively large amount of calcium during reproduction in snails are the sources of calcium and the characteristics of calcium transport

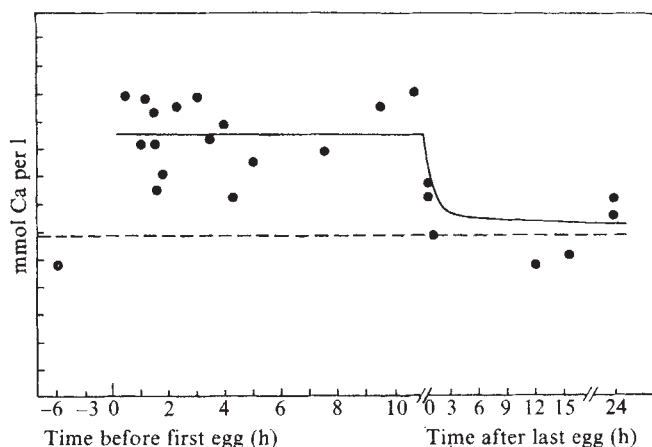


Fig. 2 Total calcium concentration of the blood of *Helix aspersa* at various stages of the egg-laying cycle. For each data point only a single sample of blood was taken from any given snail in order to avoid the lowering of calcium which otherwise may occur from trauma in laying animals (unpublished observations). Because of the great difficulty of predicting when an animal will initiate oviposition, only one point is available for the 6-h period before the appearance of the first egg. This sample represents blood calcium concentration only 2–3 h before egg shell calcification begins since it typically takes 3–4 h for a given egg to be fully calcified in passing through the entire length of the uterus. During egg shell calcification, represented on the graph by 0–11 h after the first egg laid, the blood calcium level was almost 70% higher than the normal level represented by the dotted line. This elevated blood calcium was maintained at a rather constant level as indicated by the regression equation for the line ($y = 0.05x + 9.48$). Once the last egg was released, there was a sudden drop in the blood calcium level within 3 h and near normal values were reached a few hours later.

across the uterine epithelium. These are being examined.

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Inhibition of post-implantation development of mouse blastocysts *in vitro* after cyclophosphamide treatment *in vivo*

It has been widely believed that embryos, after treatment in the preimplantation period, either die before implantation or survive to term without being malformed^{1,2}. But evaluation of the embryolethal effect of cyclophosphamide (CPA) before implantation in the rat has revealed effects of the early treatment on both embryo and mother around and after implantation³. Further investigations of the development of CPA-treated preimplantation rat embryos are impossible because rat embryos have not been cultured successfully *in vitro* beyond the time of implantation. On the other hand, routine culture of mouse embryos *in vitro* during the same period has been standardised^{4–6}. Such systems are sufficiently sensitive for investigations of genetically-determined abnormalities^{7,8} and the effects of *in vitro* exposure to ultraviolet and X irradiation^{9,10},

Table 1 Changes in blood of *Helix aspersa* during egg laying

	Normal	Egg-laying
*Calcium:		
total (mmol l ⁻¹)	5.89 ± 1.08 (14)	9.93 ± 1.29 (21)
% ultrafilterable	63 ± 3 (4)	61 ± 6 (4)
Sodium:		
total (mmol l ⁻¹)	65.7 ± 2.1 (9)	64.9 ± 3.4 (4)
pH	7.80 ± 0.03 (5)	7.79 ± 0.05 (6)

Helix aspersa snails were obtained from California and kept in the laboratory in standard light conditions in groups of 20, matched for approximate size. For all data on egg-laying snails, controls were animals from the same container in which the ovipositing snails were found, thus minimising variability in the degree of hydration, availability of food, and so on. All snails were kept in the active, non-aestivating state by providing high humidity, carrots, paper and calcium carbonate. Blood was taken by puncture of the vena pulmonalis immediately anterior to the auricle using a plastic syringe after the overlying shell area was quickly and gently removed. The pH of the blood was measured with a combination micro-glass electrode (MI Electrodes) on a Beckman expanded scale pH meter. Blood was drawn into a 1-ml plastic syringe; the hypodermic needle was immediately removed and the electrode inserted deeply into the syringe body containing the blood so that the tapered body of the electrode made an airtight fit with the syringe opening. Duplicate measurements were made within 45 s of sampling and no pH drift was observed over more than 5 min. (*Helix* blood freely exposed to air becomes more and more alkaline from loss of CO₂.) For atomic absorption analysis of total blood calcium, a 10-μl sample of blood was diluted with 1.17% (w/v) La₂O₃ in 5% (v/v) HCl (to prevent interference by elements such as aluminium, sulphate and phosphate) and then measured with a Perkin-Elmer 107 instrument. For sodium analysis, 10-μl samples of blood were diluted with distilled water and measured by flame emission spectroscopy. Blood was filtered at 15 pounds per square inch of N₂ or 2% CO₂:98% O₂ using a Clinical Ultrafiltration unit and an Amicon Diaflo Membrane of 1,000 MW cut-off. Ultrafiltration typically started with 1.0 ml of blood and no more than 50% of the fluid was allowed to pass through before the filtrate was collected. The amount of calcium in the filtrate is called unbound (mostly ionic and some complexed) and the remainder which is unfilterable is called bound. Values are mean ± standard deviation; sample size in parentheses.

**P* < 0.01.

Table 1 Cell number of mouse embryos at implantation (day 3), 24 h after CPA treatment of the mother

Developmental stage	Dose (mg kg ⁻¹)	Cell no. per embryo	No. of determinations
Morula (control)	0	25.9 ± 4.8	18
Blastocyst (control)	0	42.3 ± 11.3	25
Blastocyst (treated)	20	34.5 ± 13.0	17
Blastocyst (treated)	40	21.2 ± 3.7*	35
Blastocyst (treated)	60	18.4 ± 4.6†	43
Blastocyst (treated)	80	15.9 ± 2.8†	63

Determinations were performed at 1400 h on day 3 as described by Tarkowski¹⁵. At this time the number of embryos per mother (7.0 ± 2.7 in 148 determinations), the ratio blastocysts per morula ($5/2$ in 148 determinations) and the mitotic index (4% = cells in mitosis expressed as percentage of total cells of the embryo) were unaffected by any of the CPA doses tested. The cell numbers of morulae from CPA-treated mothers were affected in the same way as were blastocysts. The maternal LD₅₀ for CPA was 200 mg kg⁻¹.

*Significantly lower than cell number of control blastocysts at $P < 0.01$ (Wilcoxon-test).

†Significantly lower than cell number of both control blastocysts and morulae at $P < 0.01$ (Wilcoxon test).

³H-thymidine¹¹ and metabolic inhibitors¹²⁻¹⁴. To elucidate further the action of CPA on the embryos after maternal treatment during the pre-implantation period, we have used mouse blastocysts to repeat our studies with CPA in the rat, and we have also cultured mouse blastocysts for 120 h from CPA-treated mothers. With concentrations of CPA that did not affect the morphology of blastocysts from treated mothers but only the cell number at the time of implantation, development of the blastocysts *in vitro* was inhibited in a dose-dependent manner. This system holds promise as a test for putative teratogens.

To determine the rate of lethality at term after CPA treatment 24 h before implantation, groups of 10 pregnant NMRI mice (Zentralinstitut für Tierzucht, Hannover) received a single subcutaneous injection of CPA at 20, 40, 60 or 80 mg kg⁻¹ (a gift from Asta-Werke AG) on day 2 (day 0 = the day after the mating night). At term the resorption rates for the four doses tested were 35, 78, 100 and 100%. As described for the rat³, CPA did not reduce the number of embryos per mother before and around the time of implantation but significantly decreased the number of living embryos during organogenesis. As with the rat, no malformations could be detected and the somite numbers of treated mouse embryos surviving to organogenesis were significantly lower than for controls, indicating a retardation of development of about 24 h. This could have been explained by a delay in implantation and so the exact time of implantation was determined as before³. Again as in the rat, there was no indication of delayed implantation in the treated group. But at CPA concentrations of 40–80 mg kg⁻¹ (Table 1) the cell numbers of treated blastocysts were significantly smaller than those of controls and of the 20 mg kg⁻¹ group ($P < 0.01$, Table 1). At the highest CPA concentrations (60 and 80 mg kg⁻¹) the cell numbers of blasto-

cysts were significantly smaller than those of normal morulae (Table 1). The considerably smaller cell numbers in blastocysts of treated animals is evidence for interference by CPA or one of its metabolites with embryonic development *in vivo* during the 24 h between days 2 and 3. At the same time there was histological evidence of a simultaneous inhibition of the decidual reaction of the uterus, as in the rat³.

Blastocyst transplantation has been used to show the maternal CPA treatment has a direct effect on pre-implantation rat embryos³. But with the mouse a different approach was used. Blastocysts treated *in vivo* were cultured *in vitro* in conditions such that differentiation usually proceeds as follows⁵: hatching from the zona pellucida after 36–48 h; attachment to the surface of the dish after 48 h, and outgrowth of three characteristic cell types—a trophoblast layer with trophoblast giant cells and an inner cell mass (ICM) consisting of two germ layers, ectoderm and endoderm—which is usually completed after 96–120 h. In our culture system embryos from CPA-treated mothers showed a dose-dependent inhibition of all steps of differentiation (Table 2). The percentage of embryos which underwent ICM differentiation into two germ layers was the most sensitive parameter. It was already significantly decreased at the lowest CPA concentration (20 mg kg⁻¹), which had no significant effect on the cell number of the blastocysts (Table 1). Studies of treatment *in vitro* have revealed a higher sensitivity of the ICM cells than of trophoblast cells to substances which interfere with nucleic acid metabolism¹¹⁻¹⁴.

Other investigators have reported morphological abnormalities in pre-implantation rat¹⁶ and mouse¹⁷ embryos after treatment of the mother. The morphology of early rat³ and mouse embryos was not affected by CPA as far as blastulation is concerned. But the cell number of CPA-treated blastocysts was considerably smaller than normal. This effect of CPA on *in vivo* development of pre-implantation embryos between the eight-cell (day 2) and the blastocyst stage (day 3) indicates that blastulation, the first step of morphological differentiation, is not dependent on the presence of a particular number of cells and that cells, having lost the capacity to divide at a normal rate, can still form the blastocyst cavity. The effects of CPA on subsequent development *in vitro* might be explained by a retarded clearance of the alkylating agent or one of its metabolites from the blastocyst cavity^{3,18}.

For the evaluation of the effects of teratogens on pre-implantation embryos, the *in vitro* approach has several advantages over embryo transfer for which success has been low and poorly reproducible¹⁸. The *in vitro* system requires fewer embryos, and it is faster and more precise because maternal factors and individual variations are not involved. It also gives clearcut dose-response relationships, which are usually not obtained when treated embryos are transferred¹⁹.

Our technique analyses only the direct effect of the teratogen on the embryo itself and does not allow an assessment of the influence of a disturbed maternal physiology on later development. The detection of maternal effects of the early treatment

Table 2 Effect of cyclophosphamide (CPA) treatment *in vivo* on differentiation of mouse blastocysts *in vitro*

CPA dose (mg kg ⁻¹)	No. of blastocysts (100%)	Hatching (%)	Attachment and outgrowth (%)	Extensive trophoblast growth (%)	ICM 2 germ layers (%)
0	98	97	97	96	93
20	119	89*	91	82†	61†
40	73	81	80*	71*	25†
60	135	62†	62*	50†	11†
80	50	30*	28†	22†	0†

Blastocysts were flushed from the uteri at 1400 h on day 3 (24 h after maternal treatment) and cultured in groups of 10 in medium NCTC-109 (Difco) supplemented with 10% foetal calf serum (Gibco) in plastic culture dishes at 37 °C in a humidified 5% CO₂ in air atmosphere, as described by Sherman⁵. Significance levels were determined by the χ^2 test separately for each step of differentiation by comparing the growth rates (as percentage of blastocysts cultured) at every CPA dose with the growth rate at the next lower CPA dose.

* $P < 0.05$.

† $P < 0.01$.

‡ $P < 0.001$.

still requires transplantation experiments. In our studies with the rat we were able to show an inhibitory effect of early CPA treatment on the maternal environment by transplanting untreated embryos to treated and untreated pseudopregnant foster mothers³. Furthermore, transfer experiments are always necessary to check whether *in vitro* treatment during the preimplantation period is able to induce malformations at term^{18,20}, especially because term malformations have been induced after transfer of preimplantation mouse embryos treated *in vitro* with the insecticide captan²¹.

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Carbon dioxide reversibly abolishes ionic communication between cells of early amphibian embryo

Low electrical resistance intercellular junctions have been found in a wide variety of adult tissues, both *in vivo* and in tissue culture¹ and in early embryos². Vertebrate and invertebrate adult intercellular junctions are permeable to small ions and a variety of other molecules, of molecular weights possibly up to 1,000, as shown by the movement of tracers such as fluorescein³ and transfer of nucleotides ('metabolic cooperation')³. In adult systems, such intercellular exchange is correlated with the presence of gap junctions⁴. In embryos, some form of specific junction is necessary to account for the observed electrical coupling after early cleavage stages⁵ and the presence of gap junctions has been reported^{6–8}. Since the low resistance intercellular pathway has been implicated in the control of spatial and temporal organisation during development⁹, the permeability of the embryonic junction assumes some importance. There is evidence suggesting that embryonic junctions are less permeable than adult junctions^{10–13}, which we have recently confirmed¹⁴. Our experiments¹⁴ suggested that there is selectivity in the gap junctional membrane which led us to predict that the junctional permeability would be sensitive to changes in intracellular pH. We present here results which confirm this prediction.

Electrical coupling between cells was measured in *Xenopus* embryos between the four-cell and mid-blastula stages. The embryos were continuously superfused with Holtfreter's solution (NaCl, 60 mM; KCl, 1 mM; CaCl₂, 4 mM) buffered to

the required pH with 2 mM Tris hydroxymethylaminomethane or 10 mM ADA (*N*-(2-acetamido) iminodiacetic acid) (extracellular pH change) or 40 mM NaHCO₃/CO₂ (intracellular pH change). Two glass intracellular microelectrodes (filled with 0.8 M potassium citrate) were inserted into one cell, one to record the membrane potential, E_1 , and the voltage deflection, V_1 , produced by a rectangular hyperpolarising current pulse (10^{-8} A, 1 s long, every 10 s) injected through the second microelectrode. A third microelectrode monitored the membrane potential in an adjacent cell, E_2 , together with the voltage deflection produced by current flow through the intercellular junction, V_2 . The ratio V_2/V_1 gives a measure of the efficiency of electrical coupling between the cells. All parameters were displayed on an oscilloscope and slow writing chart recorder.

Extracellular pH changes between pH 5.8 and 7.5 had little effect on membrane potential, membrane resistance or electrical coupling. Intracellular pH was lowered by superfusing the embryo with a bicarbonate buffered Holtfreter solution saturated with 100% CO₂; CO₂ crosses the membrane and forms carbonic acid which liberates H⁺, so lowering pH_i (ref. 15). Figure 1 shows the effect of exposing a 64-cell embryo to 100% CO₂; by contrast with an extracellular pH change, this brought about a rapid increase in junctional resistance, culminating in complete uncoupling of adjacent cells.

Table 1 summarises experiments on embryos between the 4-cell and blastula stages of development. In four- and eight-cell embryos lowering the intracellular pH had no effect on the coupling ratio V_2/V_1 . Treatment with CO₂ produced a change in membrane potential, of variable magnitude and sign, at all stages of development tested, suggesting that depolarisation is not the cause of uncoupling; the membrane potential changes may be caused by an effect of CO₂ on the permeability of non-junctional membranes. When CO₂ uncoupled the cells there was invariably a concomitant rise in input resistance. Since CO₂ caused little change in resistance at the eight-cell stage it is unlikely that the uncoupling is brought about by the earthing of restricted extracellular current pathway normally responsible for current flow from one cell to the next. The most reasonable conclusion is that current flow from one cell to the next is largely mediated by a specialised intercellular pathway, whose permeability is greatly reduced when the embryo is

Fig. 1 Exposing a 64-cell embryo to 100% CO₂. The pen record (B) begins with the embryo in Holtfreter solution at pH 7.5. The membrane potential was -17 mV and the coupling ratio, 0.9. Oscilloscope records of the individual electrotonic potentials V_1 and V_2 are shown above (A). At the arrow a Holtfreter solution buffered with 40 mM NaHCO₃ and equilibrated with 100% CO₂ (at pH 6.5) was admitted to the bath. The membrane potential in cell 2 decreased by 10 mV while the input resistance, recorded in cell 1, increased; at the same time electrical coupling between the cells disappeared. After 2.5 min uncoupling was complete, only capacitative artefact remains on the E_2 trace. A (b), confirms the complete absence of an electrotonic potential in the adjacent cell. The return to Tris-buffered solution rapidly restored both membrane potential and electrical coupling (c).

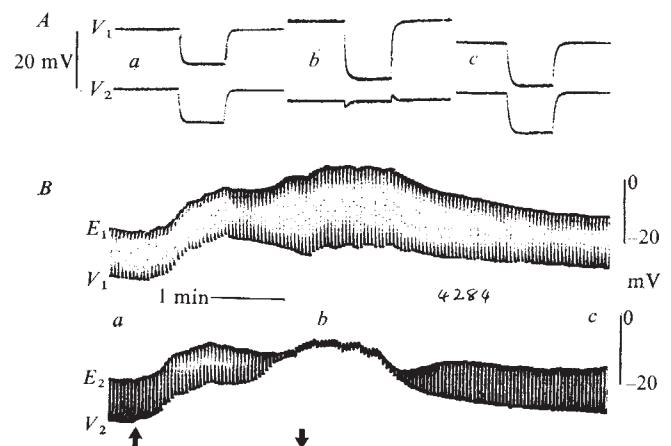


Table 1 Uncoupling induced by CO₂ at different developmental stages

	Initial resting potential (mV)	Potential during CO ₂ (mV)	Final membranal potential (mV)	Effect of CO ₂ on coupling
Stage 3 (4 cell)	20 11	6 0	8 0	None None
Stage 4 (8 cell)	11 11 10	5 6 10	5 8 13	None Partial uncoupling None
Stage 5 (16 cell)	26 16 18	10 3 5	18 15 18	Uncoupling Uncoupling Uncoupling
Stage 6 (32 cell)	21 10 8	0 0 0	24 8 10	Uncoupling Uncoupling Uncoupling
Stage 6½ (64 cell)	40 20 17 —	18 3 7 3	20 10 14 30	Uncoupling Uncoupling Uncoupling Uncoupling
Stage 7 (mid blastula)	— 20 25	3 5 5	33 25 30	Uncoupling Uncoupling Uncoupling

Membrane potential recorded at V_2 electrode; all potentials negative with respect to zero. At 4- and 8-cell stage measurements made across first cleavage plane. Uncoupling means V_2/V_1 falls to zero. No effect means normal V_2/V_1 ratio (between 0.8 and 0.9).

exposed to CO₂. The insensitivity of early cleavage stage embryos may reflect incomplete cleavage (the cells cleave every 20 min), or a change in membrane properties as the embryo enters the morula stage. In all other cases CO₂ completely abolished current flow from one cell to the next. This was always rapid and totally reversible.

Measurements of intracellular pH (using pH-sensitive micro-electrodes¹³) (in collaboration with R. C. Thomas) showed the pH to fall from a resting value of 7.7 to between 6.4 and 6.2 on exposure to 100% CO₂. The intracellular pH change followed the same time course as the uncoupling. Extracellular pH changes between 5.8 and 7.5 had no effect on the intracellular pH.

The simplest interpretation of these results is that the effect of CO₂ treatment on electrical coupling between cells of the early *Xenopus* embryo is mediated by the low intracellular pH. An effect of anoxia is unlikely, since early amphibian embryos are notoriously resistant to oxygen lack¹⁹ and we have found that treatment with 60% CO₂: 40% O₂ is equally effective. It has been proposed that the permeability of the intercellular junction is controlled by the intracellular concentration of ionised calcium¹⁶. Since Meech and Thomas¹⁷ have recently demonstrated that intracellular injection of Ca²⁺ leads to a concomitant fall in intracellular pH, the possibility that a fall in intracellular pH rather than a rise in free Ca_i was responsible for uncoupling observed in experiments such as those on the *Chironomus* salivary gland of Rose and Loewenstein¹⁸ must now be considered. The question whether in our experiments the increase in intracellular H⁺ displaces calcium from intracellular binding sites, so that intracellular calcium exerts the ultimate control remains open, although there are arguments against this possibility. We have injected iontophoretically into *Xenopus* embryo cells at the 64-cell stage sufficient calcium to bring about profound contracture of the contractile cytoplasm beneath the plasma membrane (making the free calcium level likely to be well above 10⁻⁶ M (ref. 6)) without affecting the intercellular flow of current. 100% CO₂ did not produce any sign of contraction of the contractile cortex, despite its efficacy as an uncoupling agent, suggesting that no massive release of

calcium occurs. We therefore consider that a rise in Ca_i was not primarily responsible for the uncoupling seen in *Xenopus* embryos on lowering intracellular pH.

The results described here open up a number of interesting possibilities. First it may be that the intracellular pH, rather than intracellular calcium, controls junctional permeability and similar experiments on the *Chironomus* salivary gland become important. Second, an increase in intracellular pH could cause a rise in junctional permeability and may provide a way of making the poorly permeable embryonic junction more like its adult counterpart. Finally, despite the difficulty of eliminating possible side effects of CO₂ treatment, our experiments offer, for the first time, the opportunity to test whether electrical coupling plays an essential role during development, as it should now be possible to reversibly uncouple the embryonic amphibian system and observe the developmental consequences. Experiments to answer these questions are already in progress.

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Unique cytoplasmic phosphoproteins are associated with cell growth arrest

THE intracellular molecular processes responsible for the regulation of animal cell growth are not understood. Cyclic AMP has been implicated in the arrest of cell growth (see ref. 1 for review) and it has been postulated that all cyclic AMP effects are mediated through protein kinase². Insel *et al.*³ have demonstrated with mutants that a cyclic AMP-dependent protein kinase mediates the growth inhibitory effect of cyclic AMP in S49 cells. Maller and Krebs⁴ have established that the catalytic subunit of cyclic AMP-dependent protein kinase was both necessary and sufficient to block progesterone induced meiotic cell division in *Xenopus* oocytes. The above observations suggest that a high steady-state level of a phosphoprotein which is subject to control by cyclic AMP-dependent protein kinase functions in a regulatory manner to arrest cell growth. Since cyclic AMP-dependent protein kinases are found predominantly in the cytoplasm, we investigated the phosphorylation of cytoplasmic phosphoproteins and report here that quiescent cell cytosol contains several unique phosphoproteins.

The cell line used in this study was baby hamster kidney (BHK)₂₁ clone 13. Logarithmically growing cells were brought to quiescence by incubation in serum-deficient medium (0.1% calf serum in Dulbecco's modified Eagle's medium) for 40 h.

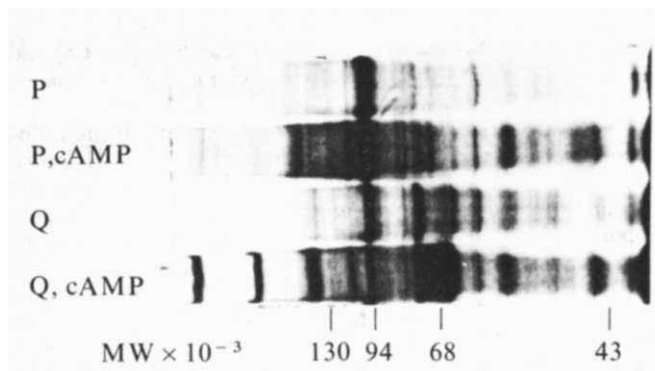


Fig. 1 *In vitro*-labelled cytoplasmic phosphoproteins in quiescent and proliferating cells. Cytosol was prepared from quiescent (Q) and proliferating (P) BHK cells in the following manner. Cells on 100-mm dishes were washed twice with phosphate-buffered saline and scraped from the plate with a rubber policeman. The cells were centrifuged at 600g and the cell pellet washed twice more with phosphate-buffered saline. The cell pellet was resuspended in homogenisation buffer (10 mM HEPES (pH 7.4), 5 mM $MgCl_2$, 25 mM KCl), and cells were disrupted in a Potter-Elvehjem homogeniser. The homogenate was spun at 100,000g for 30 min and the supernatant (cytosol) dialysed for 12 h against the reaction buffer (100 mM NaCl, 20 mM HEPES (pH 7.2), 10 mM $MgCl_2$, 1 mM EGTA) at 4 °C. Inclusion of a protease inhibitor, phenylmethyl sulphonyl fluoride, in the dialysis buffer had no effect on the electrophoretic protein profile nor on phosphorylation. Cyclic AMP-dependent protein kinase activity was examined before and after dialysis using exogenous histone as substrate: no activity was lost in either cytosol preparation during dialysis. Cytosol protein concentration was adjusted to 2.5 mg protein per ml and theophylline (2 mM), NaF (10 mM), dithiothreitol (1 mM) and cyclic AMP (1 μ M, when present) were added. The reaction was initiated by the addition of γ - ^{32}P -ATP (2 μ M, 1,000–3,000 Ci mmol $^{-1}$) and was run for 5 min at 30 °C. The kinetics of phosphorylation revealed that maximum incorporation of ^{32}P occurred by 5 min in both quiescent and proliferating cytosol proteins. The reaction was terminated by addition of electrophoresis sample buffer (1% SDS, 1% mercaptoethanol, 50 mM Tris pH 6.8, final concentrations), and boiling for 1 min. Proteins were separated by SDS-polyacrylamide slab gel electrophoresis⁵. Gels were stained with Coomassie blue in 45% methanol and 7.5% acetic acid, destained and dried. Phosphoproteins were visualised by exposure of the dried gel to X-ray film. Molecular weights of phosphoproteins were estimated by comparison with standard proteins ($MW \times 10^{-3}$ shown below autoradiogram) as described by Weber and Osborn⁷. cAMP, cyclic AMP.

At this point over 95% of the cells were arrested in G_1 phase of the cell cycle as determined by flow microfluorimetry.

Cytoplasmic extract was prepared from quiescent and proliferating cells and subjected to *in vitro* phosphorylation with γ - ^{32}P -ATP. The cytosol proteins were then separated by sodium dodecylsulphate (SDS)-polyacrylamide slab gel electrophoresis, and the phosphoproteins were identified by exposing the dried gel to X-ray film. Approximately 20 phosphoproteins were identified in cytoplasmic extracts from both quiescent and proliferating cells in the absence of cyclic AMP; most of the phosphoproteins in quiescent and proliferating cytosol were identical and phosphorylated to the same extent (Fig. 1). But a protein of molecular weight (MW) 76,000 was consistently phosphorylated only in cytosol from quiescent cells. In proliferating cell cytosol a protein of MW 96,000 was more intensely phosphorylated than in quiescent cytosol (Table 1). The pattern of Coomassie blue stained proteins in quiescent and proliferating cells was identical.

Incubation of the cytosols with cyclic AMP resulted in several striking differences in the phosphoprotein profile. Five proteins were consistently phosphorylated only in quiescent cell cytosol in the presence of cyclic AMP (Figs 1 and 2). These proteins included two that were larger than 220,000 (largest band seemed to be a doublet), and proteins of MW 140,000, 76,000 and 66,000. Surprisingly, cyclic AMP always inhibited the phosphorylation of the 96,000 MW protein by 40% (Table 1). The presence of cyclic AMP stimulated the phosphorylation

of only one protein (160,000 MW) unique to the proliferative growth state. All other cyclic AMP-stimulated phosphoproteins seemed to be in common with quiescent cell cytosol. Cyclic AMP also decreased phosphorylation of the 96,000 MW protein in proliferating cytosol (Table 1). Similar cyclic AMP effects were observed at concentrations of cyclic AMP from 0.1 to 10 μ M.

The same cyclic AMP-dependent phosphoproteins were observed when BHK cells were growth-arrested by isoleucine deprivation or serum deprivation, indicating that these phosphoproteins are associated with the quiescent state induced by two different conditions and are not simply the result of low serum in the culture medium.

The ^{32}P -labelled bands in Fig. 1 were not destroyed by RNase, DNase, hot trichloroacetic acid or 1 M hydroxylamine. Protease treatment before electrophoresis destroyed all labelled bands as did 0.4 M NaOH at 37 °C for 18 h, indicating that the ^{32}P bands represent phosphate covalently esterified to serine or threonine residues in cytosol proteins.

Wehner *et al.*⁶ have reported that density-inhibited cells contain a 110,000 MW protein which is phosphorylated in the absence of exogenous cyclic AMP. We could not observe this

Fig. 2 Quantitation of ^{32}P incorporated into cytoplasmic phosphoproteins in quiescent and proliferating cells. The dried gel from the experiment depicted in Fig. 1 was cut into 2-mm slices and counted in a liquid scintillation counter. *a*, ^{32}P incorporated into resting cell cytosol proteins in the presence (—) or absence (---) of cyclic AMP. *b*, ^{32}P incorporated into proliferating cell cytosol proteins in the presence (—) or absence (---) of cyclic AMP.

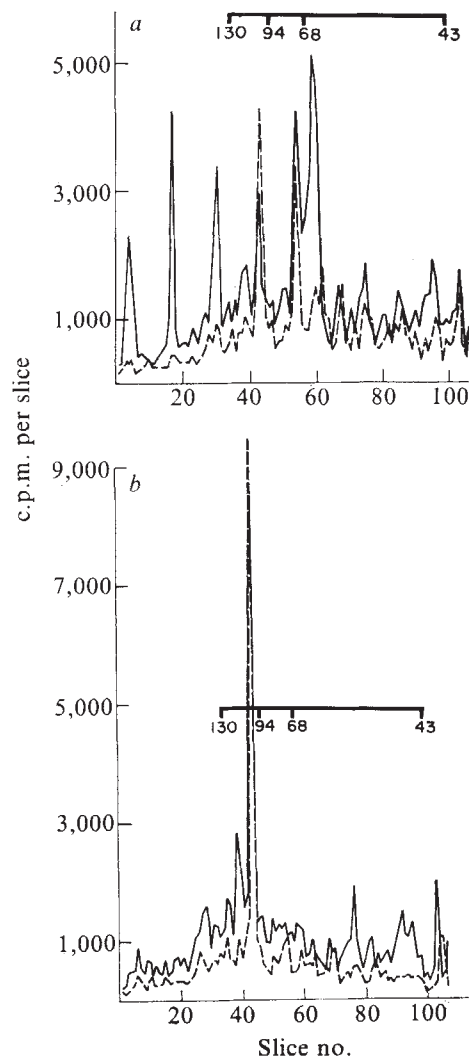


Table 1 Phosphorylation of the 96,000 MW protein in quiescent and proliferating cells and the effect of cyclic AMP

Source	³² P c.p.m. per band*
Resting cell cytosol	8,490
Resting cell + cyclic AMP	5,022
Proliferating cell cytosol	27,689
Proliferating cell + cyclic AMP	21,920

Each cytosol was adjusted to 2.5 mg of protein per ml and subjected to phosphorylation conditions as described in Fig. 1. Exactly the same amount of protein was applied to the gel in each case. After the gel was dried and exposed to X-ray film, the 96,000 MW region was cut from the gel and counted in a liquid scintillation counter.

*Average of two experiments

phosphoprotein in our studies: this may be because different cell lines were used in the two studies but important methodological differences may also explain this discrepancy. First, we used culture conditions in which the cells at the time of the experiment were at the same density but the growth state was modulated by the presence or absence of serum in the culture medium. Second, we dialysed the cytosol against the reaction solution before carrying out *in vitro* phosphorylation reaction. This brings the cytosol to specified pH and ion concentration and permits the study of cyclic AMP effects on phosphorylation. Wehner *et al.*⁶ did not dialyse the cell extract and found no cyclic AMP effects that could be related to growth. Third, we used homogenisation in a Potter-Elvehjem homogeniser to disrupt cells and prepare cytosol while Wehner *et al.*⁶ used sonication, a technique that damages membranes and disrupts cellular organelles, and carried out phosphorylation reactions on the total cellular sonicate. Thus, the relationship of the 110,000 MW phosphoprotein of Wehner *et al.*⁶ to our data is not clear.

Results from several studies (reviewed in ref. 1) have established that elevated cyclic AMP is associated with the arrest of cell growth. The data presented here establish that quiescent cells consistently contain five unique phosphoproteins, four of which are phosphorylated only in the presence of cyclic AMP, and provide a possible mechanism by which cyclic AMP can control animal cell growth. The phosphoproteins may function as regulatory proteins to stop cell growth and maintain a G₁ block. Addition of mitogenic substances such as serum, which lower cyclic AMP⁸, to the culture medium would result in the dephosphorylation of the particular phosphoprotein(s) which maintains the G₁ block and thus, transit through G₁ is permitted. Other proteins may have to be phosphorylated or dephosphorylated at other points in the cell cycle to regulate progress through the cycle. An example of this would be the 96,000 MW protein which we have found to be intensely phosphorylated in proliferating cells by a cyclic AMP-independent protein kinase. The fact that cyclic AMP inhibits the phosphorylation of this protein is consistent with its possible role in promoting cell growth.

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Tumour-dormant states established with L5178Y lymphoma cells in immunised syngeneic murine hosts

THE tumour-dormant state is one in which tumour cells persist in a clinically normal host for prolonged periods. Tumour dormancy is a potentially unstable state and may culminate in either destruction of all tumour cells or death of the host due to tumour outgrowth. We report here two systems in which tumour-dormant states could be established with transplantable lymphoma cells inoculated into specifically immunised syngeneic murine hosts. A high percentage of mice kept for long-term observation eventually developed tumours and in both systems, tumour cells could be isolated from mice during a prolonged period of clinical normality.

There are many reports describing the recurrence of solid tumours and leukaemias years after apparently successful treatment of the primary neoplasia¹⁻³. These reports suggest that residual tumour cells can persist in a dormant state during periods of prolonged clinical remission, but do not rule out *de novo* recurrence of the same type of cancer due to a long lasting predisposition of the patient's cells to malignant transformation.

Since Fisher and Fisher's first report on tumour dormancy in rats⁴, only a few animal models have been developed for the study of this state⁵⁻⁸. Tumour dormancy in experimental animals may, however, be a frequent occurrence which goes unrecognised because experiments are usually terminated before tumour dormant cells emerge to produce overt tumours. We wish to make others aware that resistance of mice to tumour-cell challenge may be associated with prolonged tumour dormancy rather than tumour cell rejection.

In our experiments, we used the methylcholanthrene-induced lymphoma, L5178Y, an immunogenic, weakly metastatic tumour of DBA/2 origin⁹. In the first system, DBA/2 mice were immunised by intraperitoneal (i.p.) injection of 10⁷ mitomycin C-treated L5178Y cells and thereby protected against the rapid outgrowth of live tumour cells inoculated i.p. 10 d later. Long-term observation of these mice revealed that 55-95% eventually developed tumours, some as late as 5 months after cell inoculation. In the experiment described here, immunised mice were killed on selected days after L5178Y cell challenge and attempts made to recover tumour cells from the peritoneal cavity and the spleen by *in vitro* culture. Additional mice from the same group were observed for extended periods for delayed tumour cell outgrowth. All non-immunised DBA/2 mice died of tumour growth within 25 d of i.p. inoculation of 5 × 10⁴ live L5178Y cells, whereas no deaths occurred in the immunised, challenged mice until day 60, and by day 210, 93% of the mice had died of tumours (Fig. 1). Table 1 shows that tumour

Fig. 1 DBA/2 females (8-10 weeks) were immunised by intraperitoneal (i.p.) injection of 10⁷ mitomycin C-treated L5178Y cells. Control mice received an equivalent amount of PBS. All mice were given 5 × 10⁴ L5178Y cells i.p. 10 d later. Shaded histogram, controls; open histogram, treated animals.

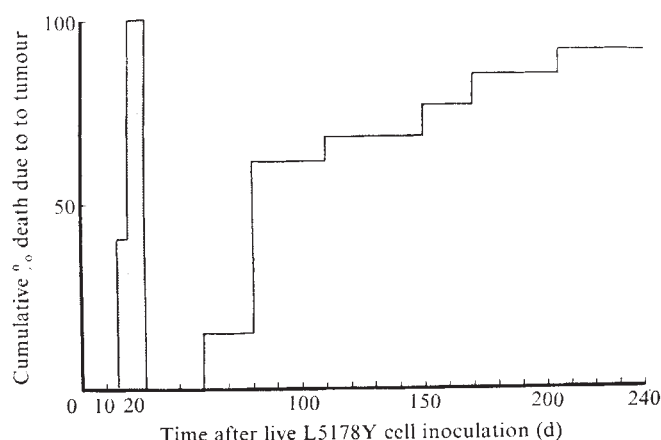


Table 1 *In vitro* isolation of tumour cells from clinically normal mice previously immunised with mitomycin C-L5178Y cells

Time after tumour cell challenge (d)	Site of tumour cell isolation (No. mice with tumour cells/total no. mice tested)	
	Peritoneal cavity	Spleen
30	3/5	0/5
49	2/2	1/2
55	4/7	3/7
Total	9/14	4/14

Clinically normal mice (see text) were killed at the times shown. Their peritoneal cells and spleens were collected, washed and single cell suspensions of each were placed into culture at various concentrations and observed for 14 d. Cultures were judged positive for tumour cell outgrowth if increasing numbers of large lymphoblastoid cells appeared and continued to proliferate when transferred into new culture vessels.

cells could be isolated from cultures of peritoneal cavity cells and of spleen cells removed from these mice during the period when they were clinically normal. (Mice were judged to be clinically normal if total body weight did not exceed 30 g; the abdomen did not seem distended; macroscopic tumour foci were not visible following killing; and the total peritoneal cell count did not exceed 1×10^7 cells.) The criterion for isolation of tumour cells *in vitro* was the appearance of increasing numbers of large lymphoblastoid cells which continued to proliferate following transfer into new culture vessels. These cells, when inoculated i.p. into normal DBA/2 mice, routinely produce ascitic tumours within 30 d, indicating that tumour cells recovered from dormant mice retain their tumorigenicity in normal syngeneic animals. An attempt was made to determine the period of persistence of tumour cells in a dormant state and we found that they could be recovered from the peritoneal cavity of clinically normal mice as long as 380 d after original tumour cell challenge.

To avoid the possible artefacts that occur when immunisation and tumour challenge are both given intraperitoneally, we used a sinecomitant immunisation procedure in our second system. DBA/2 mice were injected subcutaneously with 1×10^6 L5178Y cells and 10 d later the small (1 cm) tumour nodules were excised. Such control mice remained free of tumour throughout a 160-d observation period, and no tumour cells could be isolated from their spleen or peritoneal cells, indicating that excision of the nodule had been complete and that no metastasis had occurred from the primary site of implantation. Excision of the tumour nodule protected mice against the rapid outgrowth of 5×10^4 L5178Y cells inoculated i.p. 7 d later (Fig. 2); however, most of these challenged mice eventually developed tumours by day 160. Tumour cells could be isolated from the peritoneal cavity of many

of the immunised and challenged mice while they were clinically normal (Table 2).

Results of experiments in both systems show that specific immunisation does not lead to complete elimination of all challenge tumour cells, but rather results in the establishment of a tumour-dormant state in a high percentage of animals. During this dormant state, tumour cells which were restrained from outgrowth *in vivo* grew out rapidly when transferred to *in vitro* culture. Although immunisation with mitomycin C-treated or X-irradiated tumour cells^{10,11}, or with live tumour cells which were subsequently removed^{12,13}, has previously been reported to protect mice against the rapid growth of a tumour cell challenge, in none of these studies were the mice observed long enough for dormant cells to emerge.

Essential to the tumour-dormant state is *in vivo* restraint of tumour cell outgrowth. In our systems a single surviving L5178Y cell doubling unrestrained at its normal *in vivo* rate (~ 11 h) can produce a pronounced ascites within 30 d—an interval which represents a small fraction of the observed tumour-dormant period of clinical normality. The possible mechanisms of tumour cell growth restraint *in vivo* include: continuous tumour cell division with concurrent tumour cell destruction; cytostatic effects which greatly prolong the *in vivo* population doubling time of the

Table 2 *In vitro* isolation of tumour cells from clinically normal mice previously immunised by L5178Y tumour nodule formation and excision*

Time after tumour cell challenge (d)	Site of tumour cell isolation (No. mice with tumour cells/total no. mice tested)	
	Peritoneal cavity	Spleen
40	0/2	0/2
50	0/2	0/2
55	0/2	0/2
60	3/3	0/2
65	1/1	0/2
72	3/3	1/3
75	1/2	1/2
Total	8/15	2/15

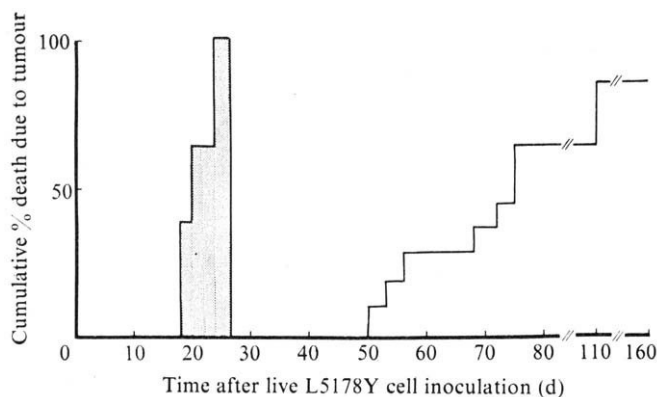
*See legend to Table 1.

tumour cells; immunoselection of a tumour cell subpopulation which grows more slowly than the original population; and sequestration of residual tumour cells in anatomical sites where they can escape destruction but still be inhibited in their division rate. Eccles and Alexander⁵ showed that tumour metastases may be maintained in a dormant state by immunological mechanisms, since immunosuppressive measures resulted in outgrowth of tumour cells.

The rapid *in vitro* outgrowth of tumour cells from peritoneal and spleen cell cultures prepared from tumour-dormant mice suggests that effector cells or soluble factors that suppress tumour cell proliferation *in vivo* may be either lost or inoperative after removal from the animal. A related observation may be the "escape from cytostasis" described by Remington and coworkers¹⁴. They found that activated macrophages could inhibit ³H-thymidine uptake by tumour cells for only 24 h after *in vitro* challenge, with renewed uptake of labelled thymidine observed thereafter. A similar tumour escape from host cytostatic mechanisms may be responsible for *in vitro* tumour cell outgrowth in our models.

Absolute identification of the origin of tumour cells emerging after the dormant period is very difficult in our syngeneic systems. We have, however, observed that tumour cells which emerged in dormant mice 80 d after challenge grow out rapidly when inoculated into normal mice but fail to grow out when inoculated into L5178Y-immunised mice, indicating that the emerging cells express L5178Y tumour-associated transplantation antigens. Additionally, DBA/2 mice have an extremely low incidence of spontaneous lymphomas and although electron microscopy of

Fig. 2 DBA/2 females (8–10 weeks) were immunised by implantation of 10^6 live L5178Y cells subcutaneously on the ventral surface. Tumour nodules were removed surgically 10 d later, and mice were challenged 7 d later by i.p. inoculation of 5×10^4 L5178Y cells. Control animals received PBS subcutaneously and 5×10^4 L5178Y cells intraperitoneally at the appropriate times. Shaded histogram, controls; open histogram, immunised animals.



L5178Y cells reveals the presence of a C-type virus, no infectious virus could be demonstrated by either S+L- or XC assays, and homogenates of L5178Y cells were not tumorigenic (unpublished results).

The two murine systems reported here are now being analysed to elucidate the mechanisms involved in the establishment and maintenance of the tumour-dormant state, with a view to identifying those events which lead to breakdown of the tumour-dormant state and development of overt neoplasia.

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Post-infection genetic resistance to avian lymphoid leukaemia resides in B target cell

NEOPLASMS in which the target for transformation is a B-dependent lymphoid cell include Burkitt's lymphoma¹ and chronic lymphocytic leukaemia² of man, bovine leukaemia³ and lymphoid leukaemia in chickens⁴⁻¹¹. Inherited resistance to the development of tumours may involve at the first level, a resistance of all cells in the body to infection by tumour viruses or, at the second level, a failure of the tumour to develop or progress in virus-infected animals¹²⁻¹⁴. In mice, the H-2-associated resistance to leukaemia is at the second level and is probably an example of immune surveillance, in which the immune system eliminates or prevents the multiplication of nascent malignant cells¹⁴⁻¹⁶. Possible effector mechanisms for immune surveillance which have been described include cytotoxic cells¹⁷, antibody¹⁸ or non-lymphoid cells^{19,20}. An alternative to immune surveillance is that genetic resistance may be expressed directly by the target

cells, involving resistance either to viral infection²¹ or to malignant transformation. We have used transfers of bursal cells from genetically resistant chickens into bursa-lymphocyte-depleted genetically susceptible chickens, and vice versa, to show that genetic resistance to lymphoid leukaemia resides in the bursal target cell, and that neither non-B cells nor cooperation between non-B cells and B cells has a role in this resistance.

The genetically resistant chicks were from East Lansing inbred line 6 subline 1 (R), and the genetically susceptible chicks from the F1 cross of East Lansing inbred lines 15 subline 1 and 7 subline 2 (S)²². These chicks are semi-allogeneic, having major histocompatibility genotypes B²/B² and B²/B², respectively. Thus, it was necessary for bursal cell transfers to be made into recipients at 3 d of age, that is, within the tolerance-responsive period, in order to ensure successful bursal reconstitution in the histoincompatible transfers²³. In other respects the transfers were as previously described for syngeneic cells⁷. Recipient chicks were treated with three doses of 4 mg cyclophosphamide (Cytosan, Mead Johnson) on days 0, 1, and 2 after hatching and received intravenous injections of bursal lymphoid cell suspensions from 10-d-old donors on day 3. The doses were 1.15×10^6 cells per recipient from susceptible donors, but only 7×10^7 cells per recipient from resistant donors, since these donor chicks from line 6₁ had exceptionally small bursas. Groups of untreated and cyclophosphamide-treated chicks served as non-transferred controls. Within 1 d of the transfers, all chicks were infected intravenously (i.v.) with $>10^4$ tissue culture infective doses of avian leukaemia virus (RAV-1), and were vaccinated against Marek's disease by intramuscular inoculation with approximately 2,000 plaque-forming units of turkey herpesvirus vaccine⁷. Tests for humoral immune function were performed at 16 weeks by inoculating killed *Brucella abortus* and sheep erythrocytes i.v. and bleeding for serum 1 week later⁷. All birds dying during the experiment were necropsied and survivors were killed and examined for lymphoid leukaemia at 33 weeks of age, using both gross and histopathological study. Table 1 includes the pooled data from two replicate experiments performed 1 month apart in which the results were similar.

Regardless of recipient genotype, cases of lymphoid leukaemia occurred only in chickens receiving bursal cells from donors of susceptible genotype (Table 1, groups 1 and 2), and no cases resulted from transfer of bursal cells of resistant genotype (groups 3 and 4). The incidence of lymphoid leukaemia in the successfully reconstituted recipients of susceptible-type bursal cells (groups 1 and 2) was lower than in the untreated susceptible controls (group 8). Successful B-cell functional reconstitution with bursal cells was achieved in almost all the syngeneic transfers (groups 2 and

Table 1 Effect of bursal cell transfer on antibody response and lymphoid leukaemia development in cyclophosphamide-treated chicks

Group	Donors Phenotype	Recipients		No. tested	No. (%) responders*	Antibody and LL among responders		
		Phenotype	Treatment			SE Titre+	Ba Titre+	%LL†
1	S	R	CY	39	15 (38)	10.4	10.3	50
2	S	S	CY	32	32 (100)	11.5	11.3	44
3	R	S	CY	20	10 (50)	8.0	6.8	0
4	R	R	CY	22	21 (95)	10.9	8.8	0
5	None	R	CY	26	0	—	—	0
6	None	S	CY	29	0	—	2.6	0
7	None	R	None	26	26 (100)	11.6	11.2	4
8	None	S	None	18	18 (100)	11.9	10.4	100

Groups of genetically resistant (R) or susceptible (S) recipients were treated with cyclophosphamide (CY) on days 0-2 after hatching and received i.v. transfers of bursal cells 3 d after hatching from 10-d-old R or S donors. At 16 weeks, all birds were injected i.v. with *Brucella abortus* (Ba) and sheep erythrocytes (SE), and bled for serum 1 week later. Results of two replicate experiments were similar and are pooled.

*Responders to both SE and Ba. In group 6, 12/29 responded to Ba with very low titres (1-5 log₂).

†Mean log₂ titre of responders to each antigen.

‡LL, lymphoid leukaemia.

4), as shown by the incidence and titres of antibody responses. Reconstitution was less effective in the semi-allogeneic transfers, being 50% in the histocompatible group (R→S) and 38% in the converse histoincompatible group 1. Nevertheless, the results for lymphoid leukaemia incidence, based on successfully reconstituted chickens only, were clear-cut. Similar results were obtained in experiments in which susceptible chicks were reconstituted with syngeneic bursal cells⁷. It seems that the chance of bursal cells becoming transformed by leukaemia virus may be somewhat reduced in some reconstituted chicks, even though there are sufficient stem cells available to repopulate the antibody-forming system.

In confirmation of our previous findings⁷, the effect of cyclophosphamide treatment was to lower lymphoid leukaemia incidence from 100% to zero in susceptible chickens (groups 6 and 8), accompanied by a near-total suppression of humoral immune responses. The very low incidence of lymphoid leukaemia in resistant chickens (4%, group 7) was reduced to zero by cyclophosphamide treatment (group 5), with total loss of antibody responses.

From the clear-cut evidence that differences in lymphoid leukaemia incidence between groups of birds with successful restoration of the bursal system are associated with the genotypes of the donors rather than of the recipients, we conclude that resistance to lymphoid leukaemia of line 6₁ chickens is conferred on them by the bursal cells and not by other cellular elements of the immune system such as thymic or thymus-derived cells or non-lymphocytes. Although it is possible that this resistance may reside in B cell-mediated specific humoral immunity alone, as may occur in feline leukaemia¹⁸, it seems more likely that an intrinsic inability of the bursal target cell to become infected or transformed is the major factor.

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Two mechanisms of migration inhibition factor induction by tumour antigens

EVIDENCE has accumulated which stresses the importance of lymphokines in cell-mediated immunity both *in vivo* and *in vitro*¹. But, the processes leading to release of lymphokines by antigen-activated lymphocytes, the mode of action of these lymphokines and their exact role in cell-mediated immunity are still not well defined. It has, however, been recognised that *in vitro* activation of immune lymphocytes by soluble antigens is macrophage dependent and that this activation is under the control of gene products of the major histocompatibility complex (*H*-2 in the mouse)². Using a Moloney murine sarcoma virus (MSV)-induced tumour system, we have shown, in agreement with reports of some other systems^{3,4}, that immune T lymphocytes required macrophages for migration inhibition factor (MIF) production and, in addition, only histocompatible macrophages could assist immune T lymphocytes for MIF release after stimulation with soluble tumour-associated antigens⁵. We report here that gene products of the *H*-2 complex regulated the macrophage-immune lymphocyte interaction for MIF release when soluble tumour extracts were used as the source of antigen. In contrast, when intact tumour cells were used as the source of antigen, a macrophage requirement was not detectable. Moreover, the direct lymphocyte-tumour cell interaction for MIF release was not under *H*-2 restriction, since allogeneic as well as syngeneic tumour cells could activate MIF release from immune lymphocytes. These findings suggest the existence of at least two different pathways for MIF production, reflecting perhaps the activation of two distinct subpopulations of T lymphocytes or the activation of T lymphocytes at two different stages of differentiation.

To assess MIF production we used an indirect Agarose droplet assay⁶. Immune spleen cells (ISC) were removed from C57BL/6N (B6) mice 12–14 d after inoculation of a regressor strain of MSV. ISC were depleted of macrophages (d-ISC) by treatment with carbonyl iron powder and magnet⁶ or by passage over rayon adherence columns⁶ or Sephadex G-10 columns⁷. To generate MIF in the presence of soluble tumour-associated antigens, B6 d-ISC were incubated with normal peritoneal exudate cells (PEC) induced by light mineral oil and treated with anti-Thy-1.2 antisera plus complement. Two sources of soluble tumour-associated antigens were used: a 3 M KCl extract from Rauscher virus-induced ascitic lymphoma (RBL-5) and a 3 M KCl extract from chemically induced ascitic lymphoma [EL4 (G-)]. RBL-5 cells carry the relevant antigens to which B6 ISC are sensitised, whereas EL4 (G-) do not⁸. Alternatively, MIF was produced from B6 d-ISC by incubation with tissue culture RBL-5 cells or cryopreserved RLV or MLV ascitic tumour cells treated with puromycin (20 µg per 10⁷ cells). 2-Mercaptoethanol at the final concentration of 5 × 10⁻³ M was added to the cultures since it was found to increase the levels of MIF production by d-ISC. Each experiment was performed in quadruplicate and repeated at least 5 or 6 times. In the culture conditions used, NSC plus RBL-5 tissue culture cells or RBL-5 cells alone were found negative for MIF production as shown in a previous report⁹.

Table 1 shows MIF production by ISC in response to 3 M KCl soluble tumour extracts of RBL-5 cells while the ISC, depleted of macrophages, failed to release detectable amounts of MIF when stimulated with soluble tumour antigen. The reaction was antigen-specific because no MIF production was observed with 3 M KCl soluble extracts of EL4 (G-). However, we were able to restore the ability of d-ISC to produce MIF by the addition of syngeneic PEC or PEC from B10 mice sharing the same *H*-2 complex, but differing at many other genetic loci from B6 mice. In

contrast, PEC from B10.A and B10.D2 mice which are congenic to B10 mice and differ only at the *H-2* locus were not able to restore MIF production by B6 d-ISC in response to the antigen. Allogeneic PEC from BALB/c mice were also unable to cooperate with d-ISC in MIF production. Similar results have been observed with BALB/c mice bearing tumours induced by a regressor strain of Moloney murine sarcoma virus (unpublished). In a previous study³ we showed that the inability of allogeneic macrophages to cooperate in MIF production was not due to a malfunction of macrophages or to an inhibitory effect in allogeneic cell mixtures, so these results indicate that the ability of macrophages to assist d-ISC in the production of MIF in response to a soluble antigen is controlled by gene products mapping in the *H-2* complex.

Since *in vivo* lymphocytes come in contact with tumour antigens in both soluble form and in insoluble form (as tumour cells), we felt it was important to examine MIF production from immune lymphocytes stimulated with intact tumour cells instead of soluble tumour-associated antigens and to compare the role of macrophages with the two forms of antigen. Table 2 shows that B6 ISC, depleted of macrophages by different techniques, were able to produce MIF when the antigen was on intact tissue culture tumour cells. Moreover, MIF was already detectable at 4 h after initiation of culture while using soluble tumour extracts. MIF production required 14–16 h in addition to the preincubation of the soluble antigen with macrophages. Since the RBL-5 cells used were from an established culture cell line devoid of macrophages and the percentage of macrophages after treatment with carbonyl iron powder plus magnet in association with rayon adherence column was about 0.1%, as evaluated by latex particle ingestion and esterase staining¹⁰, MIF production in response to intact tumour cells as antigen did not have a detectable requirement for macrophages. As *H-2* compatibility is required for the interaction between PEC and d-ISC in the induction of MIF by soluble antigen, it was of interest to determine if the same restriction held for the interaction between d-ISC and tumour cells in the generation of MIF. Table 3

Table 2 MIF production by macrophage-depleted B6 ISC in response to intact tumour cells

Treatment of ISC	Tumour Cells	
	RBL-5	EL4 (G-)
Carbonyl iron-magnet	0.37*	1.11
Rayon adherence column	0.64	0.97
Sephadex G-10 column	0.41	1.05
Carbonyl iron-magnet + rayon adherence column	0.52	1.02

B6 ISC (5×10^6), depleted of macrophages by various techniques, were mixed with 5×10^5 RBL-5 tissue culture cells or EL4 (G-) tumour cells in 1 ml of medium containing 0.5% FBS and incubated for 48 h at 37 °C in 5% CO₂ atmosphere. The supernatants, adjusted to 10% FBS, were treated as described in Table 1.

* Migration index calculated as in Table 1.

shows that d-ISC were able to produce MIF in response to tumour-associated antigens on allogeneic cells as well as on syngeneic tumour cells. At the ratio of 10:1 effector cells: tumour cell no activity was observed with normal spleen cells in the presence of allogeneic target cells, excluding the possibility of MIF release by allogeneic stimulation as has been observed with longer periods of incubation¹¹.

These results demonstrated that tumour-associated antigens stimulate MIF production by immune lymphocytes by two different mechanisms. In the first one, involving soluble tumour-associated antigens, responses occur only when the antigens are presented on macrophages sharing the same *H-2* of the mice used for *in vivo* sensitisation. In the second one, involving intact target cells, the tumour-associated antigens on the membrane of the tumour cells seem to be sufficient by themselves, independent of *H-2* products on their surface and of macrophages, to stimulate the immune lymphocytes to produce MIF. The lack of *H-2* restriction in the mechanism of direct lymphocyte-tumour cell interaction seen in this study is in agreement with other findings from our laboratory indicating that the killing of tumour cells by immune T lymphocytes as measured by 16–18 h ⁵¹Cr assay is not under *H-2* restriction^{12,13}. Plata *et al.* also obtained results showing that *H-2* restriction in the MSV system is not absolute¹⁴. The characteristics of the MIF response to soluble tumour antigens are similar to those observed in the induction phase of the immune response in other systems², in which lymphocytes recognise soluble antigens only when bound to the *H-2* products of the macrophage membrane, and our results are in accord with the 'complex antigenic determinant model' proposed by Thomas and Shevach¹⁵. On the other hand, the tumour-associated antigens presented on intact tumour cells would seem to allow stimulation of immune lymphocytes directly, without the requirement for macrophages or for similarity at the *H-2* complex. This might be accounted for by a

Table 1 Requirement of *H-2* compatible macrophages for MIF production by B6 ISC in presence of soluble tumour-associated antigens

	Source of PEC	PEC <i>H-2</i> type	3 M KCl RBL-5	3 M KCl EL4 (G-)
ISC	—	—	0.78*	0.98
d-ISC	—	—	1.07	1.05
"	B6	b	0.73	
"	B10	b	0.71	
"	B10.A	a	1.01	
"	B10.D2	d	1.07	
"	BALB/c	d	1.05	

B6 ISC (5×10^6 ml), untreated or treated by rayon adherence columns (d-ISC) and mixed with 2.5×10^5 peritoneal exudate cells (PECs) from normal B6, B10, B10.A, B10.D2 and BALB/c mice, were incubated with 3 M KCl extracts of RBL-5 or EL4 (G-) tumour cells. After 4 h at 37 °C the cell mixtures were washed and resuspended in 1 ml of fresh medium containing 0.5% foetal bovine serum (FBS) for 42 h at 37 °C in 5% CO₂ atmosphere. The supernatants, adjusted to 10% FBS, were tested on normal B6 PEC induced by light mineral oil. Two μ l droplets of Agarose medium solution, containing 8×10^5 PEC, were placed with a microdispenser (Drummond Scientific) in the centre of each well of Falcon plastic Microtest II (3040) culture plates. Each droplet was allowed to solidify at room temperature for 5 min and then 0.1 ml of stimulated or control culture supernatants was added. After 24 h at 37 °C in a humidified 5% CO₂ atmosphere the migration area images were projected, traced and measured by planimetry.

* Migration index (M.I.) where

$$\text{M.I.} = \frac{\text{Migration area with stimulated supernatant}}{\text{Migration area with unstimulated control supernatant}}$$

All the M.I. under 0.85 were considered positive as determined previously⁵.

Table 3 Ability of macrophage-depleted B6 ISC to produce MIF in presence of syngeneic as well as allogeneic tumour cells

Tumour cells	<i>H-2</i> type	Inducing agent	ISC	NSC
RBL-5	b	RLV*	0.76†	0.99
MBL2	b	MLV	0.73	1.07
EL4 (G-)	b	Benzpyrene	0.97	0.95
LSTRA	d	MLV	0.70	0.98
YC8	d	MLV	0.77	1.08
YAC	a	MLV	0.79	1.10

B6 ISC or NSC (5×10^6), depleted of macrophages by rayon adherence columns, were mixed with 5×10^5 frozen tumour cells in 1 ml of medium containing 0.5% FBS and 2-mercaptoethanol (5×10^{-5} M) for 48 h at 37 °C in 5% CO₂ atmosphere. The supernatants, reconstituted with 10% FBS, were tested as in Table 1.

* RLV, Rauscher leukaemia virus. MLV, Moloney leukaemia virus.

† Migration index calculated as in Table 1.

reaction to cell-bound antigens by a different subpopulation of lymphocytes or by lymphocytes at a different stage of differentiation, perhaps the same subpopulation of cells which are capable of cytotoxicity against intact syngeneic or allogeneic tumour target cells (which also have no macrophage requirement). Alternatively, MIF production by immune lymphocytes to soluble tumour antigens versus intact tumour cells may be in response to different antigenic specificities, only one of which requires the aid of *H-2* compatible macrophages. In any event, the lack of *H-2* restriction in direct lymphocyte-tumour cell interaction has interesting implications for the mechanisms involved with *in vivo* tumour rejection, as it has been shown previously that by immunising with allogeneic BALB/c lymphoma (LSTRA), B6 mice could be protected from challenge with a syngeneic tumour (MBL-2)¹⁶.

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Correction of human mucopolidosis II enzyme abnormalities in somatic cell hybrids

THE mouse genome has been used to correct the abnormal enzyme phenotype of a human neurodegenerative disorder and to provide information on the nature of the molecular defect. This has been demonstrated in man-mouse somatic cell hybrids using human cells derived from an individual with mucopolidosis II. Mucopolidosis II (ML II) or I-cell disease is a rare fatal childhood storage disease which is associated with deficiencies and electrophoretic abnormalities of several lysosomal hydrolases¹⁻⁴. Somatic cell hybridisation provides a methodology for examining the expression of mutant genes and for identifying and mapping genes associated with human enzyme abnormalities. We have examined the isozyme patterns of six enzymes altered in this disorder in man-mouse hybrids formed with fibroblasts from an ML II patient. These altered enzymes showed corrected patterns of expression in cell hybrids. Since several human enzymes are corrected, a defect in the post-translational processing of lysosomal enzymes is suggested. Previous studies fusing human lysosomal storage disease cells with rodent cell lines have failed to demonstrate correction of the enzyme deficiencies by the rodent genome⁵⁻⁸. We present here the first example of the correction of an abnormal enzyme

phenotype associated with a human lysosomal storage disease by fusing human and mouse genomes.

Children affected with the autosomal recessive ML II disorder show Hurler-like clinical features with growth and mental retardation and mucopolysaccharide and glycolipid storage culminating in death by age 2-6 yr (refs 1, 2). Cultured fibroblasts from ML II children show large numbers of cytoplasmic inclusions and deficiencies (2-10% of normal) of the lysosomal hydrolases β -hexosaminidase, β -glucuronidase, β -galactosidase, α -galactosidase, α -mannosidase, arylsulphatase, and α -fucosidase^{3,4}. Many of these enzymes are found at elevated levels in culture media and in serum^{4,9}. Altered electrophoretic phenotypes have been observed for β -hexosaminidase¹⁰ and several hydrolases excreted from ML II cells¹¹. We have reported altered phenotypes for β -hexosaminidase, acid phosphatase₂, α -mannosidase_B, esterase-A₄, and adenosine deaminase-d in cultured ML II fibroblasts¹². These abnormalities provide markers for determining if the defect can be corrected by the addition of normal genetic material through cell hybridisation.

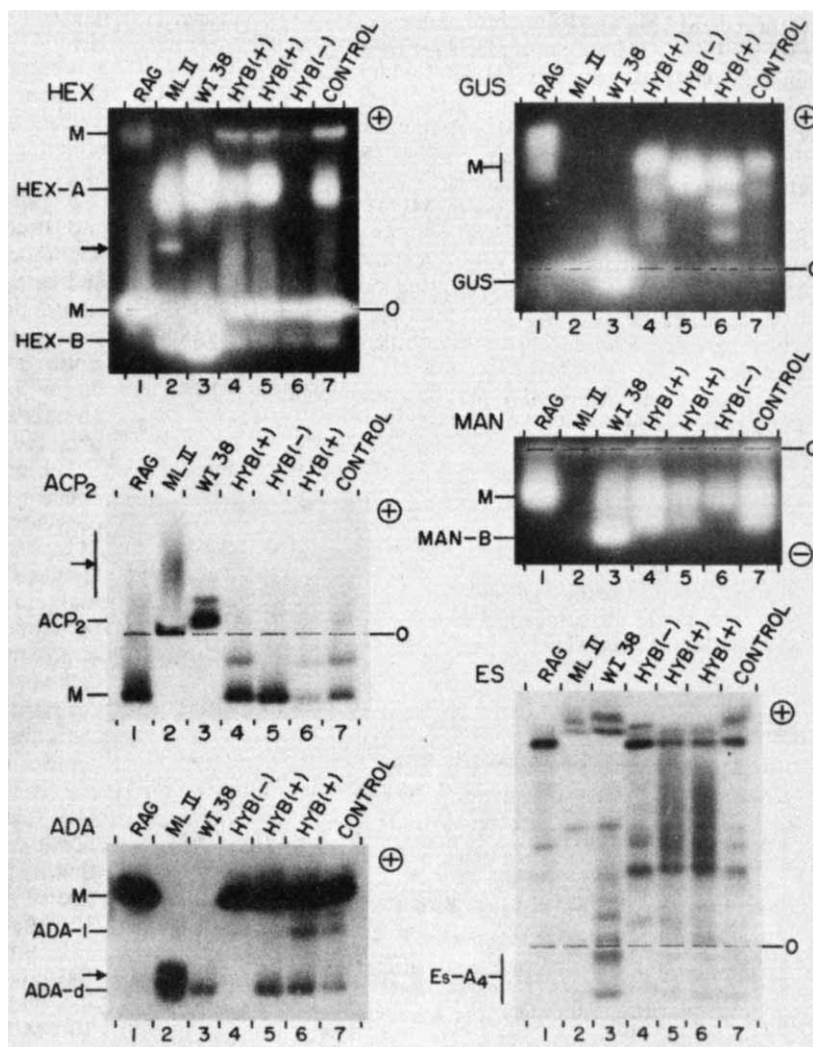
Mucopolidosis II fibroblasts (GM 1006, Human Genetic Mutant Cell Repository) were established from a female patient (L.T.) with ML II symptoms and multiple lysosomal enzyme deficiencies^{12,13}. ML II fibroblasts were hybridised with two mouse cell lines: RAG (HPRT⁻) and LM/TK⁻, using Sendai virus as described previously^{14,15}. Thirty-six independent clones were isolated consisting of 20 ML II $\times$ RAG hybrids (ICR) and 16 ML II $\times$ LM/TK⁻ hybrids (ICL). Hybrid clones appeared with a frequency of about 10⁻⁴. Primary hybrids were selected, cloned, expanded and collected as described previously¹⁴. Enzyme assays of hybrids were accomplished at early passages^{3,4} to maximise the number of human chromosomes retained in hybrids.

Enzymes with altered electrophoretic patterns in ML II fibroblasts were examined after cell hybridisation. Previously it was reported that β -hexosaminidase (HEX), acid phosphatase₂ (ACP₂), and adenosine deaminase-d (ADA-d) have altered electrophoretic banding patterns in ML II fibroblasts^{10,12}. The normal and ML II electrophoretic patterns of HEX, ACP₂, and ADA-d in human fibroblasts are compared in Fig. 1. Mobility of these enzymes in ML II $\times$ mouse cell hybrids was examined and compared with control hybrid patterns. Figure 1 shows that in ML II hybrids HEX, ACP₂, and ADA-d were identical to controls. Thus, β -hexosaminidase showed normal mobility of the human HEX_A and HEX_B bands and failed to demonstrate the altered HEX band seen in ML II fibroblasts. Human ACP₂ was identified in hybrids predominantly as the man-mouse ACP₂ intermediate heterodimer and revealed normal electrophoretic mobility and expression. Human ADA-d was also identical to the ADA-d band in control hybrids.

Enzymes severely deficient in ML II fibroblasts were also examined in cell hybrids. Figure 1 demonstrates that the lysosomal enzymes β -glucuronidase (β GUS), α -mannosidase (MAN_B), and esterase-A₄ (ESA₄) from extracts of ML II fibroblasts are weakly detected on starch gels. But human β GUS, MAN_B, and ESA₄ in ML II $\times$ mouse cell hybrids showed levels of expression on starch gels similar to control hybrids (Fig. 1). Human chromosomes are eliminated in man-mouse cell hybrids, so not every hybrid clone retained all the lysosomal enzyme markers. But, in each of the 36 hybrid clones examined, at least one of the lysosomal enzymes was retained and demonstrated the normal phenotype. A similar correction pattern was not observed by co-cultivation of mouse and ML II cells or by mixing of cell homogenates. The mouse enzyme pattern was never altered in hybrid clones.

It is possible that the chromosome on which the ML II gene is located is lost in these cell hybrids. Loss of the ML II chromosome might result in the corrected expression of the affected enzymes. Evidence supports complementation of the ML II defect since in 36 independent hybrids normal phenotypes of the enzymes were observed in each hybrid clone. The human chromosome complement in hybrid clones was determined

Fig. 1 Starch gel electrophoretic patterns of lysosomal enzymes in ML II $\times$ mouse cell hybrids. Enzymes shown include *N*-acetyl- β -hexosaminidase (HEX); acid phosphatase₂ (ACP₂); adenosine deaminase (ADA); β -glucuronidase (GUS); lysosomal α -mannosidase (MAN_B); and esterase-A₄ (ESA₄). Channels (1) RAG, mouse parental cell line; (2) ML II cultured fibroblast line GM 1006; (3) normal human fibroblasts, WI-38; (4-6) ML II $\times$ mouse cell hybrids; (7) control man-mouse hybrids showing normal positive human enzyme expression. Control hybrids were formed with WI-38 or other normal human fibroblasts fused to RAG cells¹⁸. HYB(+) indicates hybrid clones positive for the human enzyme and HYB(-) indicates negative human enzyme expression. Arrows indicate the altered HEX, ACP₂, and ADA-d, bands in ML II fibroblasts. The ML II extract for HEX was concentrated to demonstrate the altered HEX band. Differences in mobility of the rodent GUS band in hybrids is due to genetic variation in the mouse parental cells previously reported²¹. Starch gel electrophoretic procedures and enzyme staining were as described previously¹².



by examining 35 enzyme markers representing each of the human chromosome linkage groups^{15,16}. Although individual hybrids did not retain all human chromosomes, the entire genome was represented when all hybrids were taken together. This evidence suggests that the ML II defect was present in hybrids, but was complemented by the mouse genome.

Correction of ML II enzyme defects was also tested by examining linkage relationships of affected enzymes in cell hybrids. The negative expression of a deficient enzyme could result from either the loss of the chromosome encoding the enzyme or deficiency due to the presence of the ML II mutant gene. This was tested by examining segregation patterns of lysosomal enzymes with other enzymes assigned to the same chromosome. Concordant segregation was examined for ESA₄ linked to LDH_A on chromosome 11 (ref. 14); HEX_A linked to MPI, PK, and IDH_M on chromosome 15 (refs 17, 18); and MAN_B which we have assigned to chromosome 19 with GPI and PEPD (ref. 16). The ESA₄, HEX_A, and MAN_B lysosomal enzymes in hybrids revealed normal segregation patterns with their linked markers (Table 1). Thus when the lysosomal enzyme was absent, other markers on the same chromosome were also absent. These normal segregation patterns of affected enzymes and their linked markers suggest further the correction of the ML II enzyme phenotype.

Cell hybridisation studies with other human lysosomal storage disorders have not demonstrated correction of the deficient lysosomal enzymes. Thus in Tay Sachs-rodent hybrids, the deficient human HEX_A enzyme is not demonstrated⁵. Sandhoff-rodent hybrids do not express the deficient human HEX_A and HEX_B bands^{5,6}. Fabry-hamster cell hybrids fail to express

detectable human α -galactosidase⁷, and hybrids with mannosidosis fibroblasts do not express human lysosomal MAN_B (ref. 8).

There is evidence to suggest that ML II may be associated with a deficiency in the metabolism of sialic acid coupled glycoproteins. Several lysosomal enzymes from ML II cells including HEX, ACP₂, and MAN_B seem to be excessively sialylated^{11,12}. Cultured ML II fibroblasts have been reported to contain high levels of sialic acid¹⁹ and are severely deficient for an acid sialidase^{19,20}. It is not known if this sialidase deficiency represents the primary ML II lesion, however.

Complementation of ML II enzyme defects in man-mouse hybrids is indicated since (1) the enzymes HEX, ACP₂, and ADA-d had normal electrophoretic patterns in ML II hybrids;

Table 1 Segregation of lysosomal enzymes with linked enzyme markers in ML II $\times$ mouse hybrids

	+/+	+/-	-/+	-/-
ESA ₄ /LDH _A	24	0	3	9
HEX _A /MPI, PK, IDH _M	20	1	0	15
MAN _B /GPI, PEPD	26	1	1	8

Human gene assignments of lysosomal enzymes determined from man-rodent somatic cell hybrid studies were reported previously¹⁴⁻¹⁸. Values are numbers of independent hybrid clones scored positive (+) or negative (-) for expression of enzyme markers. The few discordant clones observed were similar to numbers in control hybrids and are accountable due to different sensitivities of enzyme stains and/or chromosome breakage. Enzyme markers for each hybrid clone were determined from the same cell homogenate.

(2) β GUS, MAN_B, and ESA₄ showed normal levels of expression; (3) the ESA₄, HEX_A, and MAN_B enzymes showed normal linkage relationships; and (4) all the human chromosomes were represented when all hybrids were considered. This evidence suggests that the ML II mutation was present and that the mouse genome can correct the multiple enzyme abnormalities of the ML II disorder.

Correction of enzyme defects in ML II hybrids shows that these enzyme abnormalities are not defects in the primary structure of individual enzymes. Rather, these results are consistent with this disorder resulting from a defect in the post-translational processing of multiple lysosomal enzymes which can be corrected by the mouse genome in man-mouse hybrids.

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Calcium ion regulates chemotactic behaviour in bacteria

CHEMOTAXIS is the process by which bacteria travel to higher concentrations of attractant or lower concentrations of repellent. Its study has attracted wide interest as an example of the stimulus-response network since bacteria are the most primitive of living creatures. Behaviour in peritrichous bacteria is indeed simple: bacteria alternately swim smoothly and tumble, which results in random reorientation for the next swim¹. Chemotaxis occurs by the increasing tendency of bacteria to tumble when headed in the 'unfavourable' direction and by increasing inclination to swim when the bacteria are headed in the 'favourable' direction (such as towards higher attractant concentrations)^{1,2}. Tumbling is caused by clockwise rotation of flagella and swimming by counter-clockwise rotation³. Addition of repellent to bacteria causes tumbling; addition of attractant causes swimming^{4–6}. Understanding how the switch that controls direction of flagellar rotation is controlled and how the controlling parameter is regulated by changes in attractant or

repellent concentration is necessary to understand chemotaxis. Here I present evidence that for *Bacillus subtilis* the free concentration of internal Ca²⁺ ion controls direction of flagellar rotation (high concentrations bringing about tumbling; low concentrations, swimming) and that repellents, which cause tumbling when added to bacteria, work by increasing the flow of Ca²⁺ ion across the plasma membrane.

B. subtilis was grown in minimal medium, filtered, washed and suspended at about 1.7×10^7 bacteria per ml. A23187, an antibiotic that transports divalent cations across membranes and hence tends to equilibrate the free concentrations of these cations in aqueous compartments separated by membranes⁷, was sometimes added as a 1:1000 dilution from a stock in ethanol: 0.1% ethanol itself did not affect behaviour. Samples of 4 or 9 μ l were then placed, sometimes in a blind experiment, on a washed microscope slide and the bacterial behaviour observed at the top of the drop.

The purpose of the experiment was to regulate the internal concentration of Ca²⁺ ion, which is present in very low concentrations because it is normally extruded from bacteria⁸, to see whether the ion might play a role in controlling behaviour. Buffered Ca was added to a basic buffer (Table 1). By adding high concentrations of the chelating agent, EGTA, and CaCl₂ in the correct concentrations, one could stably maintain low concentrations of free Ca²⁺ ion. To minimise depletion of cellular Mg, EGTA was chosen because of its low stability constant for Mg²⁺ ion ($10^{1.0}$) (ref. 9).

In absence of A23187 in 10^{-4} M Ca²⁺ ion, the bacteria swam erratically¹⁰. As the concentration of free Ca²⁺ ion was lowered, the bacteria swam progressively more smoothly. In absence of Ca²⁺ ion, they only tumbled occasionally¹⁰. In fact, it was possible in blind experiments to distinguish, although sometimes with difficulty, the decrease in tumbling between 10^{-4} M and 10^{-5} M, 10^{-5} M and 10^{-6} M, 10^{-6} M and 10^{-7} M, 10^{-7} M and 10^{-8} M, and 10^{-8} M and none.

In presence of A23187, behaviour was dramatically altered. Bacteria became incessant tumblers at 10^{-7} M and higher Ca²⁺ ion and, as without A23187, nearly incessant swimmers at 10^{-8} M and lower Ca²⁺ ion (Table 1; and see ref. 10). Furthermore, strong attractant, 10^{-3} M alanine, 10^5 -fold above its threshold¹¹, did not cause swimming in bacteria in 10^{-7} M Ca²⁺ ion and A23187. Finally, with no A23187, strong repellent, 3.2×10^{-6} M pentachlorophenol, 32-fold above its threshold⁶ did not cause tumbling if the external Ca²⁺ ion was 10^{-8} M or lower.

These results indicate that internal Ca²⁺ ion is likely to regulate the direction of flagellar rotation. Ca²⁺ ion is presumed to bind, with K_d of 10^{-7} to 10^{-8} M, to the switch that controls

Table 1 Effect of regulation of cytoplasmic Ca²⁺ ion using an ionophore and Ca buffer

[Ca ²⁺] _{free}	[EGTA] _{total}	[Ca ²⁺] _{total}	Behaviour in A23187
10^{-4}	10^{-4}	2×10^{-4}	Tumble
10^{-5}	10^{-3}	9.94×10^{-4}	Tumble
10^{-6}	10^{-3}	8.63×10^{-4}	Tumble
10^{-7}	10^{-3}	6.13×10^{-4}	Tumble
10^{-8}	10^{-3}	5.935×10^{-4}	Swim
0	10^{-3}	0	Swim

Suspension buffer contained the following components: 5×10^{-3} M piperazine-N, N'-bis(2-ethane sulphonate) pH 6.9, 3×10^{-2} M KCl, 5 mM sodium lactate, 0.05% glycerol, 3×10^{-4} M chelex-100 treated (NH₄)₂SO₄, and EGTA CaCl₂ as above. A23187 was used at 1.37×10^{-6} M. The concentration of free Ca²⁺ ion was calculated from the equation²¹ $[EGTA]_{total} = ([Ca]_t/K[Ca^{2+}]_{free}) - 1/K + [Ca]_{total} - [Ca^{2+}]_{free}$, using an apparent stability constant (K) for Ca-EGTA of $10^{6.8}$ (ref. 9). Bacteria were grown in minimal medium containing 10^{-2} M Na lactate and 0.1% glycerol to 75 Klett units (filter 66), about 2.6×10^8 bacteria per ml. Experiments were carried out as described in ref. 10 except that observation was made at the top of the drop, not near the slide, since bacteria tended to stick to the glass.

direction of flagellar rotation. When the switch is bound with Ca^{2+} ion, tumbling (clockwise rotation) is favoured; when unbound, swimming is favoured. Normally the bacteria successfully keep it out since even in 10^{-4}M external Ca^{2+} ion the bacteria do not tumble incessantly. But A23187 readily conveys it into the cell since even 10^{-7}M Ca^{2+} ion makes the bacteria perpetual tumblers. Repellent is assumed to bring about a transient depolarisation of the membrane^{6,6} and to increase its permeability to Ca^{2+} ion since, in absence of A23187, no tumbling response ensues if the external Ca^{2+} ion concentration is low enough (see above).

Similar results were obtained in a previous study¹⁰ when EGTA was added to *B. subtilis* in A23187. But, when Mg as well as Ca was removed from cells using A23187 and EDTA, then incessant tumbling ensued. One explanation¹² is that Mg^{2+} ion and Ca^{2+} ion are antagonists at the switch. Because internal Mg content is very high (20–40 mM)¹³, the fractional change in the concentration of Mg^{2+} that occurs during chemotactic stimulation is probably very small; in the absence of Ca^{2+} , Mg^{2+} binds to the switch and the cells swim smoothly. In absence of either ion, the switch stays in the tumbling (clockwise) rotation mode.

There is strong evidence that Ca^{2+} ion has a universal role in the control of biological processes. It regulates direction of swimming by *Paramecium*, for instance, when *Paramecium* strikes a barrier, the membrane depolarises and Ca^{2+} ion enters and causes a change in the direction that the cilia point. Thus, *Paramecium* backs up^{14–16}. (This mechanism is similar to the way repellents of *B. subtilis* act; attractants act differently¹².) When light strikes rhodopsin, Ca^{2+} ion flows out of the discs in which rhodopsin is housed into the cytoplasm and there brings about depolarisation of the plasma membrane to begin transmission of the nerve impulse to the brain¹⁷. Arrival of a nerve impulse to muscle leads to release of Ca^{2+} ion from sarcoplasmic reticulum to bring about muscle contraction and its return allows muscle relaxation¹⁸. Intracellular Ca^{2+} ion, which is affected by extracellular Ca^{2+} ion, regulates insulin release from the islets¹⁹. Variations in intracellular Ca^{2+} ion, whose fluxes across the plasma membrane of liver are affected by α -adrenergic hormones, regulate phosphorylase and glucose release²⁰. This process is repeated again and again. This special regulatory role for Ca^{2+} ion may have been chosen early in evolution; it seems to be the controlling element in the most primitive of sensory systems, chemotaxis in bacteria.

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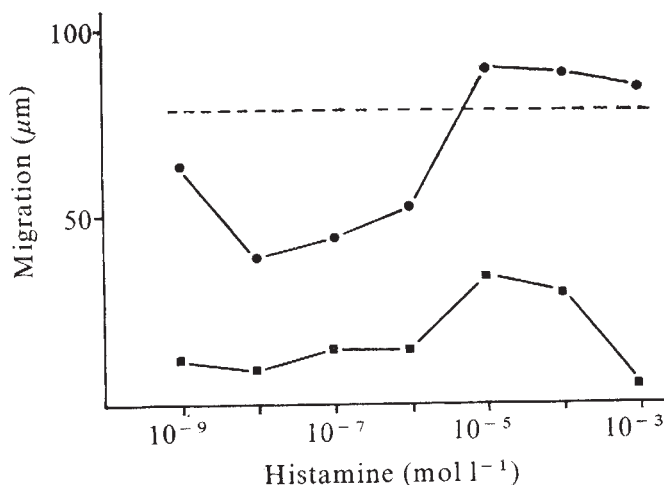
Histamine induces release of an eosinophil immobilising factor from mononuclear cells

LIBERATION of factors chemotactic for eosinophil granulocytes is often associated with the release of histamine from mast cells and basophils. This is true for the eosinophil chemotactic factor of anaphylaxis (ECF-A), which is secreted together with histamine from mast cells and basophils¹. It also applies to the generation of chemotactic factors by the activation of complement (C), which leads to the liberation of anaphylatoxin². Therefore, it was of interest to study whether histamine had any effect on the migration of eosinophils towards chemotactic attractants. We describe here a factor which inhibits the migration of eosinophil granulocytes and which is released from mononuclear cells upon incubation with histamine.

Eosinophils were induced in guinea pigs by intraperitoneal injections of a crude extract of *Ascaris suis*, 4 mg twice weekly for at least 2 weeks. Four days after the last injection the cells were collected by peritoneal lavage. The cell suspension contained between 30 and 70% eosinophils, remainder being almost exclusively mononuclear cells. Two cell types could be separated by centrifugal elutriation with a Beckman elutriator rotor³ to yield an eosinophil rich fraction of 90–97% purity, and an eosinophil poor fraction of mononuclear cells with 1–2% eosinophils.

The eosinophils showed a good chemotactic response to normal human serum which had been treated with baker's yeast (50 mg per ml serum) for 1 h at 37 °C to activate complement (C-activated NHS). They were also attracted to casein at concentrations of 0.5–2.0 mg ml⁻¹ and, to a lesser extent, to histamine, which exhibited an optimal cell response at a concentration of 10⁻⁸ mol l⁻¹ (Fig. 1). To

Fig. 1 The effect of histamine at various concentrations on the migration of guinea pig eosinophils of 40% purity towards C-activated NHS. The upper compartment of the chemotaxis chamber contained 3×10^6 cells per ml Hank's balanced salt solution (HBSS), to the lower compartment was added per ml HBSS either 50 μ l of C-activated NHS (dashed line), or histamine (■) or 50 μ l C-activated NHS plus histamine (●). Chemotaxis was assayed in a modified Boyden chamber using micropore filters of 8 μ m pore size (Schleicher & Schüll, Göttingen, Germany). The leading front of migration into the filter was measured¹⁵.



analyse the influence of histamine on the chemotactic migration towards C-activated NHS, histamine at various concentrations was added to the attractant in the lower compartment of a chemotaxis chamber. When the cell preparation contained less than 50% eosinophils, two effects were observed (Fig. 1). Concentrations of 10^{-4} to 10^{-5} mol l $^{-1}$, which themselves induced chemotactic migration, increased the distance migrated by the eosinophils. At lower concentrations, however, histamine caused a dose dependent inhibition of eosinophil migration, which was most pronounced at 10^{-8} mol l $^{-1}$. In contrast, when 90% pure eosinophils were employed in the assay, only the enhancing effect at high concentrations but no inhibition of migration at low concentrations was discernable (Fig. 2). This finding suggested that histamine did not inhibit the eosinophils directly, but induced the release of an immobilising factor from cells other than eosinophils. To test this possibility further the mononuclear fraction of peritoneal cells devoid of eosinophils was incubated with and without histamine at 10^{-8} mol l $^{-1}$ for 1 h at 37 °C. The cells were then centrifuged, and eosinophils of 90% purity were suspended in the supernatant and assayed for chemotactic migration towards C-activated NHS. As depicted in Fig. 3, there was some inhibition of eosinophil migration by the 1 h supernatant of mononuclear cells incubated without histamine. However, a marked inhibition of migration by 80% was found when histamine was present during the incubation. The immobilising effect could not be attributed to histamine, since histamine at 10^{-8} mol l $^{-1}$ did not reduce the mobility of pure eosinophils and at higher concentrations was weakly chemotactic itself (Fig. 2).

Furthermore, the histamine antagonists diphenhydramine and metiamide (Smith, Kline and French), although capable of blocking the histamine induced release of the immobilising factor from mononuclear cells, did not affect the inhibition of eosinophil migration when added together with the mononuclear cell supernatant. The immobilising factor was not cytotoxic for eosinophils: at the end of the 2-h incubation period of eosinophils with the mononuclear cell supernatant more than 97% of the cells were found to be viable as tested by trypan blue exclusion. The effect could not be explained by the release of a chemotactic factor

Fig. 2 The effect of histamine at various concentrations on the migration of more than 90% pure eosinophils towards C-activated NHS. C-activated NHS 50 μ l per ml HBSS (dashed line), histamine only (■), C-activated NHS plus histamine (●).

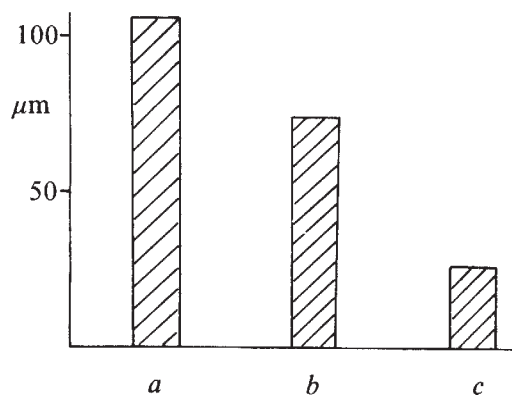
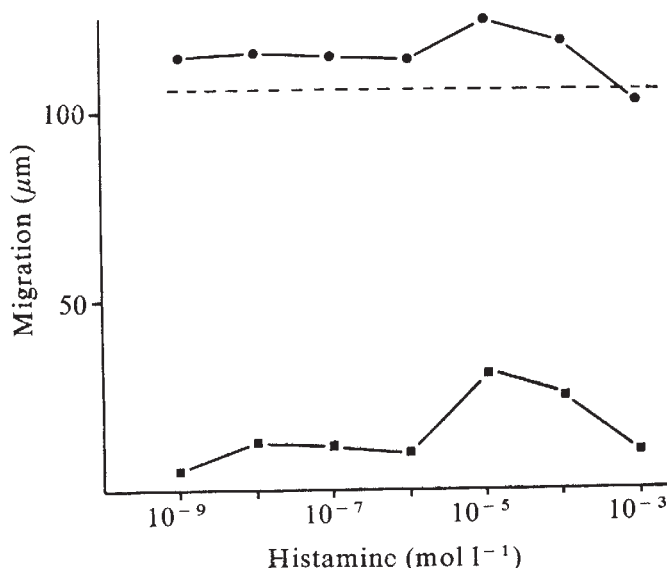


Fig. 3 The migration of 90% pure eosinophils suspended in HBSS a, the supernatant of the mononuclear cell fraction incubated for 1 h at 37 °C in the absence b and in the presence c of 10^{-8} mol l $^{-1}$ histamine. The columns represent individual data from a representative experiment.

from the mononuclear cells upon histamine treatment, since the supernatant when added alone to the lower compartment of a Boyden chamber did not exhibit chemotactic activity.

We conclude therefore that histamine at a concentration around 10^{-8} mol l $^{-1}$ stimulated mononuclear cells to release a factor which immobilised eosinophils. From a column of Sephadex G-10 the activity was eluted with the total volume indicating that its molecular weight was below 1,000. This precludes its identity with the neutrophil immobilising factor^{4,5}, which has a molecular weight of 5,000. Preliminary results indicate that the eosinophil immobilising activity is released from the nonadherent fraction of mononuclear cells and that it affects the eosinophils and not the chemotactic factor.

Various effects of histamine on the function of cells have been described. Aside from an inhibitory effect on the secretory function of mast cells and basophils^{6,7} it inhibited the destruction of target cells by cytotoxic T lymphocytes⁸, it reduced the number of plaque forming cells in an *in vitro* primary⁹ and secondary¹⁰ immune response, and it suppressed the production of the migration inhibitory factor MIF from immune lymphocytes¹¹. What is unusual about the effect of histamine described here is the stimulatory action on the secretory function of mononuclear cells and the low histamine concentration at which this effect is observed.

It has been disputed whether histamine itself is chemotactic for eosinophil granulocytes. Like others¹² we were able to induce increased migration of eosinophils towards a concentration gradient of histamine, but this differed only slightly from the increased random mobility and was considerably less pronounced than the attraction by C-activated NHS, casein or ECF-A. It has been suggested that the chemotactic activity of histamine towards eosinophils was an *in vitro* phenomenon which could not be observed *in vivo*¹³. Others, being unable to detect any chemotactic attraction, nevertheless, observed immobilisation of eosinophils at the site of an injection of histamine¹⁴. It is conceivable that this effect was induced by the inhibitor of eosinophil migration described here. We suggest that the accumulation of eosinophils observed in certain allergic diseases and in nematode infections may be the result of chemotactic attraction and inhibitor-induced trapping. Furthermore, it seems possible that the inhibitory functions of histamine described by others⁸⁻¹¹ are as well mediated by

inhibitors released from mononuclear leukocytes under the influence of histamine.

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Dark noise in retinal bipolar cells and stability of rhodopsin in rods

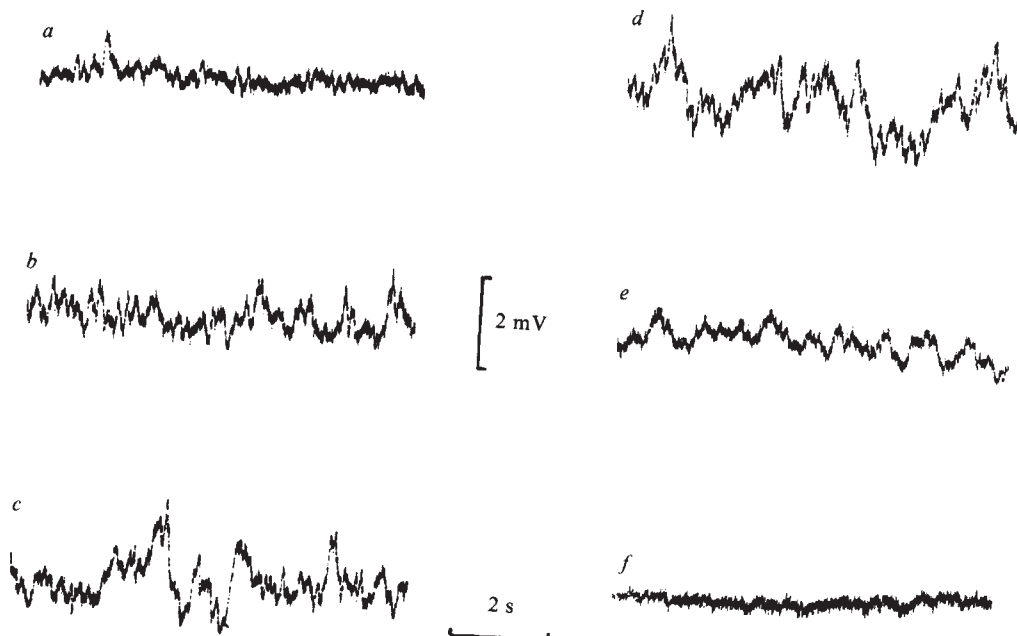
FOR the dark-adapted human observer, the absolute visual threshold has been estimated to be the effective absorption of 5–10 photons in an area covered by some 500 rods¹. Although the quantum statistics of light enter as a factor which determines the frequency of seeing weak light stimuli^{1,2}, it has been suggested that there is 'noise' in the visual system which ultimately limits the sensitivity of the eye^{3,4}. Each rod in the human eye contains about 10^8 rhodopsin molecules, and if the 'noise' arises from events in the rod indistinguishable from the effects of light, there must be an extremely low probability of

spontaneous change produced in any single rhodopsin molecule (or at sites within the rod disk membrane leading to an elementary voltage change in the rod). We have analysed voltage fluctuations in bipolar cells as a probe of rod activity in the dogfish retina. A component of the noise has been identified as photon noise, superimposed on dark noise arising from photon-like events. This part of the dark noise has a large temperature dependence (Q_{10} about 8), suggesting thermal isomerisation of rhodopsin. The rate constant, extrapolated to 37 °C, would correspond to one isomerisation in 30 s in a human rod, similar to estimates from the absolute threshold for human vision.

Using intracellular microelectrodes, we have obtained recordings from bipolar cells in the dark-adapted retina of the dogfish, *Scyliorhinus canicula*. This retina contains primarily rod photoreceptors. The bipolar cells, which depolarise with light^{5,6}, have a high flash sensitivity indicative of a large dynamic voltage gain at the rod-bipolar synapse⁶. Voltage fluctuations in the bipolar cells in darkness and during steady light were analysed for their power spectral distribution.

As shown in Fig. 1, the voltage noise increased from its dark level after presentation of light in the range 2×10^{-3} to $1 \text{ photon s}^{-1} \mu\text{m}^{-2}$. The power spectrum of the noise in complete darkness and during dim steady light consists of two components, one with a half-power point near 1.3 Hz and the other near 7 Hz (Fig. 2). Several features of the cell's response to light suggest that the low-frequency component of the noise arises from fluctuations in the rate of photons absorbed by the bipolar cell's rod pool ('photon noise'), superimposed on a noise present in the dark. The spectrum of the response to a dim, brief flash of light had power distributed over a similar range of frequencies as the low-frequency component of the noise spectrum (Fig. 2). At low light levels (less than $0.02 \text{ photon } \mu\text{m}^{-2} \text{ s}^{-1}$), the mean depolarisation produced by steady light is proportional to the light intensity. The contribution to the total noise variance, obtained from the area under the low frequency component, increases linearly with light intensity at low light levels, as expected if the underlying statistics of the source are Poisson. It was further found that the peak amplitude of the elementary event underlying the low frequency component during steady dim light was the same as that obtained from an analysis of the fluctua-

Fig. 1 Voltage fluctuations in a dogfish bipolar cell in darkness and during steady full-field illumination by blue-green light. Mean light flux density at 495 nm ($\text{photons s}^{-1} \mu\text{m}^{-2}$): a, dark; b, 1.9×10^{-3} ; c, 7.4×10^{-3} ; d, 2.1×10^{-2} ; e, 0.92; f, 8.5. (See ref. 6 for methods of voltage recording and light calibration.) Membrane potential in the dark -47 mV ; total noise variance 0.0310 mV^2 (corrected for electrode noise observed outside the cell). Mean depolarisation and noise variance during steady light were for each intensity illustrated (in order of increasing intensity) 0.34 mV , 0.103 mV^2 ; 1.1 mV , 0.163 mV^2 ; 1.8 mV , 0.227 mV^2 ; 7.2 mV , 0.101 mV^2 ; 9.5 mV , 0.0196 mV^2 . The highest intensity illustrated produced saturation of the voltage response. Peak depolarisation of the cell in response to a flash of light was 30 mV . Note that the noise variance reached a maximum value and then fell as light intensity increased. Temperature 17°C .



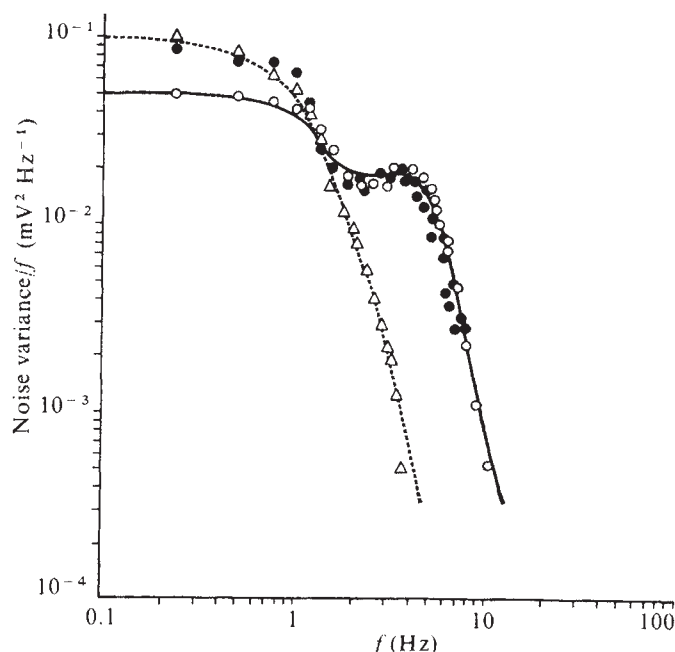


Fig. 2 Power spectra of voltage noise in darkness (○) and during steady dim light (●), compared with the spectrum of the response to a dim flash (△, the modulus squared of the Fourier transform of the flash response). Analogue data, recorded on an FM tape recorder, were filtered by a low pass filter with a cut-off frequency of 100 Hz, and sampled at 256 Hz. Spectra, weighted by a Hanning function, were computed from data blocks containing 1,024 points using the fast Fourier transform. The spectra shown are the averages obtained from 32–76 s of record and have been corrected for the electrode noise (mainly $1/f$ noise). The spectrum of the response to a flash of light represents the average spectrum of 12 responses to 15-ms flashes delivering $0.01 \text{ photons } \mu\text{m}^{-2}$, which gave a mean response of 2.3 mV. The spectrum of the dark noise has been subtracted and the result arbitrarily scaled to the lowest frequency points for the spectrum in dim steady light ($4 \times 10^{-3} \text{ photons s}^{-1} \mu\text{m}^{-2}$, mean depolarisation 0.33 mV). Noise variance in the dark attributable to 'photon-like' events = 0.032 mV^2 . Noise variance in dim steady light attributable to 'photon noise' + 'photon-like' events 0.082 mV^2 . Membrane potential in the dark -54 mV ; maximum peak depolarisation with bright light 23 mV; temperature 23°C .

tions in response to brief flashes of dim light⁷. This provides strong evidence that the same Poisson process is involved. The estimated peak amplitude of the response produced in the bipolar cell by one photon absorbed within its rod pool has an average value of 0.25 mV.

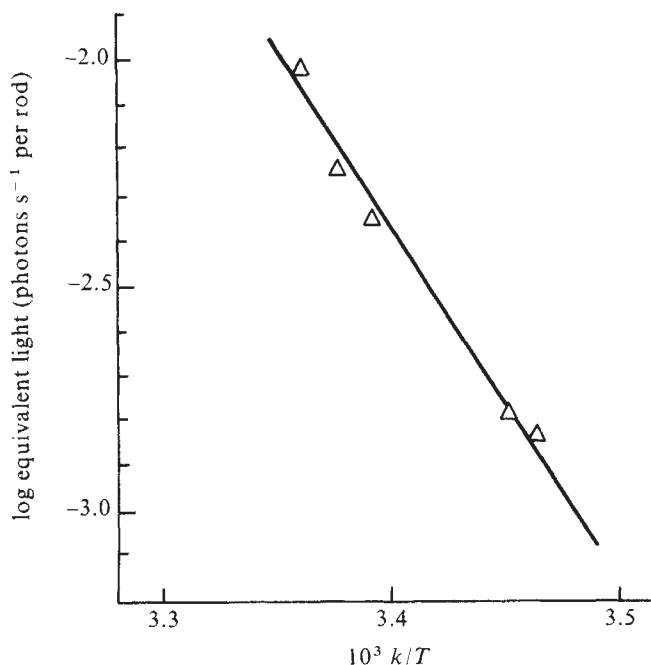
The response of photoreceptors to flashes of dim light can be described by equations of the same form as the impulse response of an electrical network consisting of a chain of low pass filters^{8,9}. It is assumed that the narrow band character of the low frequency component of bipolar cell noise arises from the properties of the rod response rather than the filter characteristics of the rod–bipolar synapse. In that case, the low frequency component of the dark noise could arise from events in the rod indistinguishable from the effects of light. The 'dark light', which would be the photon equivalent for the low frequency component of the dark noise, can be obtained from the intercept on the light intensity axis in a plot of variance against light intensity. For the cell illustrated in Fig. 2, the 'dark light' would be equivalent to a photon flux density of $2 \times 10^{-3} \text{ photon } \mu\text{m}^{-2} \text{ s}^{-1}$. This would correspond to an event equivalent to one photoisomerisation in a rod every 160 s, if the effective collecting area for a dogfish rod is $3 \mu\text{m}^2$ (ref. 6).

The 'dark light' was found to be strongly temperature dependent. An activation energy of 36 kcal mol^{-1} was

calculated from an Arrhenius plot (Fig. 3) of the mean rate of spontaneous events per rod as a function of absolute temperature, giving a Q_{10} of 7.8 at a temperature of $20\text{--}30^\circ\text{C}$. The activation energy associated with the dark light is significantly higher than for the processes governing the kinetics of the response of photoreceptors to flashes of light; 10 kcal mol^{-1} ($Q_{10} = 1.8$) for turtle cones⁸ and 16 kcal mol^{-1} ($Q_{10} = 3$) for rat rods⁹. This would make it unlikely that such processes occur spontaneously at the requisite rate. If the dark light arose from spontaneous changes in rhodopsin molecules, these changes might be of two kinds, thermal denaturation or thermal isomerisation. The products of the former are denatured opsin and 11-*cis*-retinal¹⁰, whereas for the latter they are opsin and *trans*-retinal, as in photoexcitation¹¹. The rate constant at 20°C would be $1.2 \times 10^{-11} \text{ s}^{-1}$, based on Fig. 3 and an estimate of 3×10^8 rhodopsin molecules per dogfish rod. Applying absolute reaction rate theory¹², we find that the Gibbs free energy of activation $\Delta G^\ddagger$ for the process underlying the dark light is 32 kcal mol^{-1} , and the entropy of activation $\Delta S^\ddagger$ is $13 \text{ cal mol}^{-1} \text{ degree}^{-1}$. The entropy of activation is very much less than for thermal denaturation of rhodopsin¹⁰. We conclude, therefore, that denaturation occurs at a comparatively insignificant rate in the eye over the temperature range of $15\text{--}25^\circ\text{C}$ and/or it does not give rise to responses in rods which resemble photoresponses.

The activation energy of 36 kcal mol^{-1} , determined from the dark noise, lies between the value of 26 kcal mol^{-1} for thermal *cis-trans* isomerisation of the chromophore, retinal, in a non-polar solvent, as determined by Hubbard¹³, and an estimate of about 48 kcal mol^{-1} for the activation energy for thermal isomerisation of rhodopsin^{14,15}. The estimate of 48 kcal mol^{-1} for rhodopsin is not based on direct measurement but derives from the assumption that there is a fixed minimum energy required for absorption of

Fig. 3 Arrhenius plot of the dark rate of spontaneous events in a dogfish rod, equivalent to photoisomerisation, against the reciprocal of the absolute temperature. The ordinate is the log of the mean rate of effective photon absorption by rods which would be required in order to give 'photon' noise variance in the bipolar cell equal to the variance in the dark associated with the low frequency component of the noise (see text). An effective collecting area of $3 \mu\text{m}^2$ for a rod in the tapetal region of the retina has been assumed⁶. The slope of the line corresponds to an Arrhenius activation energy of 36 kcal mol^{-1} .



a photon¹⁵⁻¹⁸, which would correspond to the thermal activation energy for isomerisation. This would, however, predict a very much larger energy of activation for *cis-trans* isomerisation of retinal than was observed by Hubbard¹⁵ (see also ref. 18). We conclude tentatively that the present results are consistent with thermal isomerisation of rhodopsin. Extrapolation to 37 °C would give a rate constant of $3.5 \times 10^{-10} \text{ s}^{-1}$. Denton and Pirenne³, and Barlow⁴, have concluded that in the human the rate constant for thermal isomerisation of rhodopsin must be less than 10^{-9} s^{-1} in order to account for the reliability of detection at absolute threshold. The low rate of spontaneous isomerisation of rhodopsin is one of the features of the visual system underlying the ability to detect the absorption of a few photons.

It is not yet clear whether the second component of the noise in bipolar cells reflects synaptic noise arising from fluctuations in the release of transmitter at the rod-bipolar synapse¹⁹ or noise generated more distally in the photoreceptor, possibly from interaction of an intracellular transmitter with the surface membrane of the rod²⁰⁻²².

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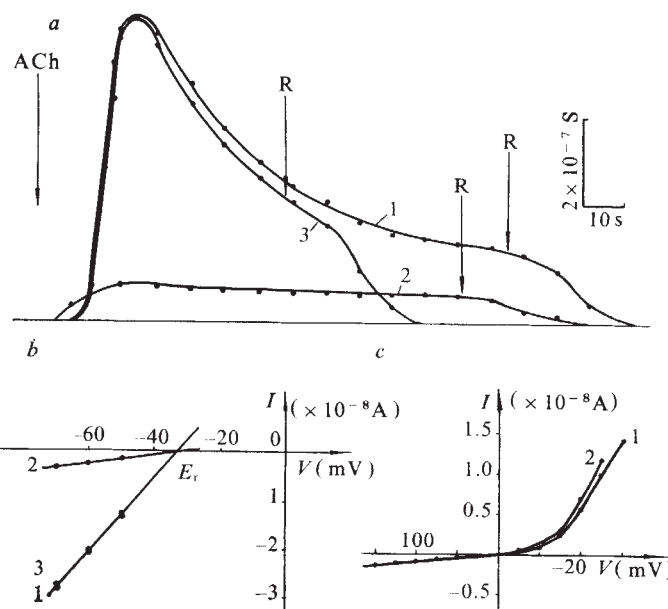


Fig. 1 The effect of disulphide bond reduction by DTT and reoxidation by DTBCh on soma membrane of *Limnaea stagnalis* neurone. 1, Control; 2, after 14 min treatment with DTT $1 \times 10^{-3} \text{ M}$; 3, after 15 min reoxidation by DTBCh $1 \times 10^{-4} \text{ M}$. *a*, The time course of membrane conductance change induced by ACh $2 \times 10^{-6} \text{ M}$. The times of ACh and Ringer solution (R) addition are marked by arrows. *b*, Voltage-current plots of the neurone response to ACh $2 \times 10^{-6} \text{ M}$. The reversal potential, E_r (obtained by extrapolation), does not change after the neurone exposure to DTT. *c*, The steady-state voltage-current curves of electroexcitable membrane. In (*a*) and (*b*) membrane potential was held at the resting potential (RP) level and a three-level voltage sequence (RP + 10 mV, RP, RP - 10 mV) was applied. ChRM conductance values were calculated according to current changes produced by membrane potential shifts in equal periods of time (dots in (*a*)) and in Figs. 2 and 3). Curves were drawn by hand. In (*c*) membrane potential was held at RP level and a serial depolarising and hyperpolarising voltage steps (10 mV) were applied. Impulse duration, 5 s. Potassium outward currents were measured at the end of the steps.

the most reliable proof of the specificity of modifying agent action¹². We present here a detailed study of the action of DTT on nicotinic AChRs of completely isolated *Limnaea stagnalis* neurones in order to clarify the ability of various cholinergic ligands to protect the receptor disulphide bonds against reduction by DTT.

Identified giant neurones from the right and left parietal ganglia of molluscs were isolated by treating brain preparations with trypsin and then Pronase, and separated mechanically with tungsten microneedles and glass micropipettes^{13,14}. Nerve cells were placed into the chamber (effective perfusion volume 0.2 ml, rate of perfusion 15–20 ml per min) and continuously perfused with *Limnaea* Ringer solution containing NaCl 100 mM, KCl 1.6 mM, CaCl_2 4 mM, MgCl_2 1.5 mM, Tris-hydroxymethylaminomethane 0.12 g l^{-1} , and HCl to pH 7.5. All the drugs were dissolved in this medium and applied to the neurone membrane by passing constant-flowing (gravity feed) solution. Each neurone was impaled with two microelectrodes (2.5 M KCl filled). The perfusing medium was connected with the virtual ground of an operational amplifier through the agar bridge. Drug action was estimated using the voltage-clamp technique according to the membrane conductance change at the resting potential level.

Application of DTT 1×10^{-4} – $1 \times 10^{-3} \text{ M}$ for 2–15 min markedly depressed responses to a standard ACh concentration (Fig. 1*a*), while the reversal potential of ACh-responses and the steady-state current-voltage curve of electroexcitable membrane did not change (Fig. 1*b* and *c*, respectively). The sensitivity of neurone membrane to ACh was restored after treatment with dithiobischoleline¹⁹ (DTBCh) which is known to oxidate thiol groups (Fig. 1*a, b*).

The reversible inhibition of cholinergic membrane

Acetylcholine receptor conformational transition on excitation masks disulphide bonds against reduction

KARLIN and Bartels¹ found that dithiothreitol (DTT) inhibits the responses induced by acetylcholine (ACh) in the *Electrophorus electricus* electroplax preparation and that 5,5'-dithio-bis-(2-nitrobenzoic acid) completely restored the membrane sensitivity to ACh. These results illustrate the importance of disulphide bonds for acetylcholine receptor (AChR) function, and work on other preparations with nicotinic AChRs gave similar results²⁻⁹. Several facts suggest that DTT affects AChRs specifically; (1), the significance of the quaternary ammonium group in the molecules of alkylating and acylating agents for the rate of their reaction with the AChR reduced by DTT¹⁰; (2) changes in pharmacological specificity seen in the modified receptor^{5,7,10}; (3) the decrease in the slope of the dose-response curve to carbamylcholine expressed as the Hill plot¹¹; and (4) the increase in *d*-tubocurarine affinity to the AChR active site⁵. If we could protect the receptor against chemical modification by an agonist or antagonist, this would be

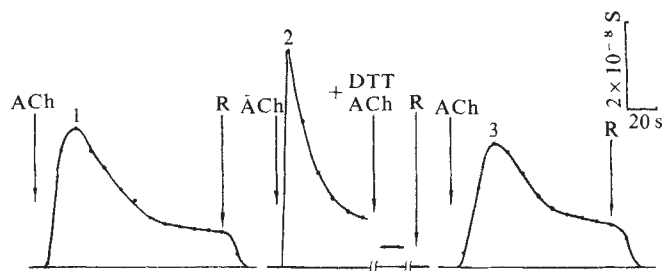


Fig. 2 Protection of ChRM against the DTT effect by a high ACh concentration. 1 And 3, response of the neurone to ACh 2×10^{-6} M before and after exposure to DTT 1×10^{-3} M for 2 min in the presence of ACh 5×10^{-5} M. 2, Response to conditioning ACh concentration; the duration of pretreatment with ACh was about 2 min.

(ChRM) by DTT could be converted into irreversible inhibition by application of an SH-specific alkylating agent (such as *N*-ethylmaleimide (NEM), any quaternary ammonium *N*-alkyl derivative of maleimide, or bromoacetylcholine (BrACh)) on the neurone. On the native neurone BrACh caused a strong reversible increase in membrane conductance, being only 3–5 times less active than ACh; quaternary ammonium *N*-alkyl derivatives of maleimide were very weak agonists (3–4 orders less effective than ACh) and NEM did not excite ChRM at all. It was not possible to restore the ACh-sensitivity to the initial level with DTBCh after application of an alkylating agent to DTT-treated neurone membrane. These results underline the important role of disulphide bonds in the ACh-induced conductance changes of neurone membrane which has been shown for both electroplax and skeletal muscle preparations.

BrACh seems to cause the irreversible depolarisation of electroplax membrane pretreated with DTT¹⁵, but this has not been observed with *Aplysia* neurones⁹ and we have never observed this effect with *Limnaea* neurones. In addition, the responses of neurone membrane to ACh and BrACh decreased approximately to the same extent after DTT pretreatment. These data suggest that BrACh cannot activate reduced AChRs of mollusc neurone membrane.

We have tested cholinergic antagonists and agonists for the ability to protect ChRM against DTT-induced modification. The specific competitive nicotinic antagonist *d*-tubocurarine (1×10^{-3} M) completely blocked response to supramaximal ACh concentrations (up to 1×10^{-4} M) but did not prevent the modification of ChRM by DTT. In contrast to this unexpected finding high ACh concentrations effectively protected ChRM against the action of DTT (Fig. 2).

One explanation of the difference in *d*-tubocurarine and ACh protecting abilities may be that the molecules of these two cholinergic agents interact with different sites in AChR so that ACh produces a steric hindrance for DTT attack on disulphide bond, but *d*-tubocurarine does not. Alternatively, binding of ACh molecule to the AChR active site might evoke a conformational transition of AChR which would protect the disulphide bond from reduction; binding of *d*-tubocurarine would not induce such a conformational change.

In order to test the first hypothesis we compared the protecting ability of two pairs of drugs: tetramethylammonium (TMA)–phenyltrimethylammonium (PhTMA) and decamethonium–diphenyldecamethonium. The first agent of each pair is a cholinergic agonist, the second drug (a structural analogue of the other) does not excite but blocks the receptors effectively. Neither PhTMA nor diphenyldecamethonium protected ChRM against DTT-reduction, while their exciting analogues, TMA and decamethonium, partially blocked the effect of DTT (Table 1). It seems highly unlikely that the drugs with such similar chemical structures as TMA and PhTMA or decamethonium and diphenyldecamethonium should bind to different sites on AChR. A direct correlation between the activity of agonists and the ability to prevent the modifying action of DTT was found (Table 1). ACh,

Table 1 Effect of cholinergic ligands on AChR inactivation induced by DTT

Ligand	Relative efficiency of exciting action*	Concentration used to protect against DTT (M)	Extent of protection†	Relative efficiency of protection against ‡
ACh	1	1×10^{-5}	0.60 (5)	1
TMA	0.02	1×10^{-4}	0.64 (3)	0.107
Decamethonium	0.0014	1×10^{-4}	0.28 (4)	0.047
PhTMA	0.0001	1×10^{-3}	0 (6)	0
Diphenyl-decamethonium	0	1×10^{-3}	0 (2)	0
<i>d</i> -Tubocurarine	0	1×10^{-3}	0 (4)	0

* Calculated by the ratio of the equieffective concentrations relying upon increase in ChRM conductance.

† Calculated by the formula $(c-b)/(a-b)$ where a = response to a standard ACh concentration in control; b = response to the same ACh concentration after DTT treatment; c = response to standard ACh concentration after treatment with DTT + protector. No. of experiments given in parenthesis.

‡ Calculated by the ratio of protection extent and protector concentration.

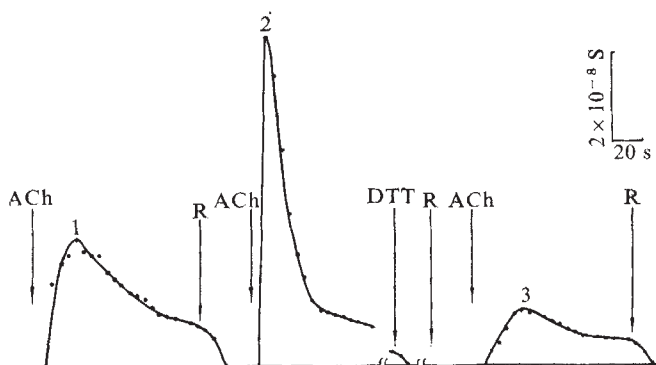
TMA and decamethonium therefore seem to protect AChRs by inducing a transition in AChR to another conformation when the agonist binds with the active site; in the new conformation the disulphide bond becomes inaccessible to DTT action.

Thus our results suggest that it is the receptor molecule alone that DTT modifies to cause inactivation of ChRM.

In all experiments involving the use of agonists as protectors DTT was added into the perfusing medium when almost all the AChRs were desensitised (Fig. 2). Presumably the AChR active site conformational change protecting the disulphide bond against reduction corresponds to the desensitisation but not to the excitation. This suggestion seems quite likely according to data on conformational transition of AChRs revealed by fluorescent dyes in membrane fragments of electric organs^{16,17} in the presence of high (desensitising) agonist concentrations. To test this possibility we compared the DTT action in the presence and absence of ACh after 2 min exposure of the neurone to a high ACh concentration. In the latter case ACh was washed out with physiological solution containing DTT when desensitisation was complete (Fig. 3). A considerable fraction of AChRs was shown to be still in desensitised state during the first 1.5–2 min after ACh was removed from the perfusing solution. In these conditions DTT inactivated ChRM as strongly as it did in the complete absence of a protector. Apparently, for successful protection, the AChR–agonist complex must exist.

We therefore conclude first, that AChR does not lose its affinity

Fig. 3 Inactivation of ChRM by DTT treatment immediately after removal of ACh (1×10^{-5} M) from the perfusing solution. Control experiments ensured that responses to a standard ACh concentration were very much reduced in 1.5–2 min after washout of ACh 1×10^{-5} M (a result of desensitisation). 1 And 3, Responses to ACh 2×10^{-6} M; 2, response to conditioning ACh concentration (1×10^{-5} M).



to agonists in the desensitised state; and second, the active site of the receptor in both the activated (non-desensitised) and the desensitised states can experience a conformational transition when interacting with an agonist molecule; the disulphide bond is hidden as a result of this process.

Desensitisation of *Limnaea* neurone membrane has been shown not to be due to changes in the system of ionic transport through the excited ChRM¹⁸. Considering these data and our results we suppose that the desensitisation of *Limnaea* neurone membrane is due to the specific state of a unit coupling the excited active site of AChR and ChRM ionophore.

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Cellular regulation of an allosteric modifier of fish haemoglobin

ADENOSINE TRIPHOSPHATE (ATP) is the major organic phosphate in the red blood cells of a wide variety of fish species^{1–4}. Its role as an allosteric modifier of fish haemoglobins is similar to that of 2,3-diphosphoglycerate (2,3-DPG) in mammalian red blood cells, in that it decreases the affinity of haemoglobin for oxygen^{5,6}. After exposure to the anoxia of high altitude or in certain conditions of anaemia, mammals increase red blood cell 2,3-DPG to facilitate oxygen unloading at the tissues⁷. By contrast, eels have been shown to decrease erythrocyte organic phosphate (that is, ATP) and increase haemoglobin–oxygen (Hb–O₂) affinity when acclimated to low environmental oxygen⁸. To test if this phenomenon was expressed in another fish species, we acclimated *Fundulus heteroclitus*, a euryhaline minnow, to hypoxic conditions. Our finding that this fish also lowered red blood cell ATP by as much as 40% (Fig. 1) suggests that this is a general response to hypoxia among water-breathing vertebrates.

The mechanism by which hypoxic fish lower red blood cell ATP *in vivo* is unknown. We predicted that if the control mechanism was directed at the erythrocyte level, then fish red blood cells should decrease ATP, *in vitro*, in anoxic conditions. Consistent with our *in vivo* observation, we found that anaerobic *F. heteroclitus* red cells significantly lowered their ATP levels *in vitro* (Fig. 2). Since the nucleated erythrocytes of fish possess mitochondria, we reasoned that this response may be mediated by way of a decrease in oxidative phosphorylation. This hypothesis was supported when aerated cells were incubated in the presence of low concentrations of cyanide. This inhibitor of aerobic respiration reduced intracellular ATP to levels similar to those found in the anoxic cells (Fig. 3). Thus, it seems that one reason fish have retained a functional oxygen-consuming electron transport system in the

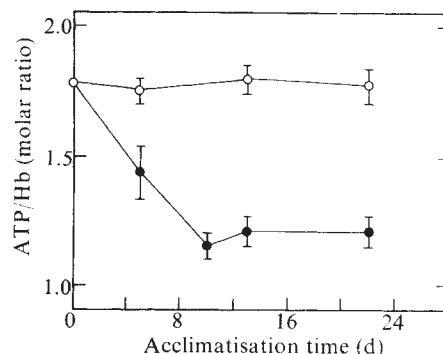


Fig. 1 Time course of acclimatization of *F. heteroclitus* to hypoxic (●) and normoxic (○) conditions at 22 °C. Dissolved oxygen values were 1.5–2.0 p.p.m. for hypoxic and 8.5–9.0 p.p.m. for control fish. Red blood cell ATP for this experiment and all others described in this paper were determined using the firefly luciferase assay⁹. All points represent averages of 6–7 fish. Bars indicate $\pm$ s.e.m.

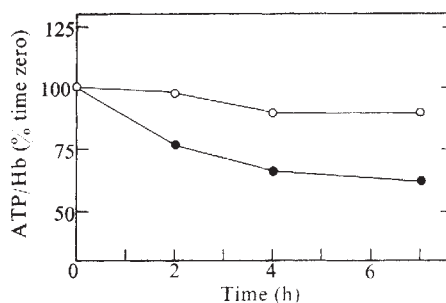
mitochondria of their red blood cells is to control the levels of haemoglobin allosteric effector.

Glycolytic inhibition experiments are consistent with the cyanide data (Fig. 3). With iodoacetate, an inhibitor of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), reducing equivalents from glycolysis are not available for oxidation by the mitochondrial electron transport chain and less pyruvate is oxidised in the tricarboxylic acid (TCA) cycle. Consequently, red blood cells exposed to this inhibitor lower their ATP to levels nearly equal to those of cyanide-poisoned cells. In the presence of fluoride, which inhibits enolase, the red blood cells show concentrations of ATP only slightly lower than control levels since reducing equivalents are formed during the earlier GAPDH reaction which can then fuel mitochondrial oxidative phosphorylation.

Assuming that iodoacetate and fluoride are acting as specific inhibitors in the red blood cells, these data indicate that the small amount of reducing equivalents from glycolysis would be nearly identical to that resulting from a fully functioning TCA cycle. Since no data exist on the relative contributions of glycolysis and the TCA cycle to the production of reduced nicotinamide adenine dinucleotide in fish erythrocytes, one cannot be certain of the intracellular potential to oxidise pyruvate. In fact, the information reported here suggests that the TCA cycle may play a substantially smaller role in fish red blood cell mitochondria compared with other tissues. We are currently investigating this possibility.

Since a physical dependence exists between oxygen solubility and water temperature (oxygen concentration is 12.5 p.p.m. at 10 °C compared with 7.5 p.p.m. at 30 °C), our observation that *F. heteroclitus* lowers red blood cell ATP when acclimated to elevated temperatures (group I, Table 1) prompted us to ask if this response was triggered by reduced oxygen (as in our hypoxic experiments), increased temperature, or both of these variables. Therefore, fish were acclimated to various temperatures (10 °C, 22 °C, and 30 °C), but with oxygen concentration maintained

Fig. 2 ATP/Hb molar ratio in *F. heteroclitus* red blood cells incubated in anaerobic (●) and aerobic (○) conditions. Cells were washed three times in PBS (pH 7), and then maintained in a medium containing physiological concentrations of inorganic salts and glucose buffered at pH 7.5 as described elsewhere⁹. Values are from duplicate determinations which did not vary by more than 6%.



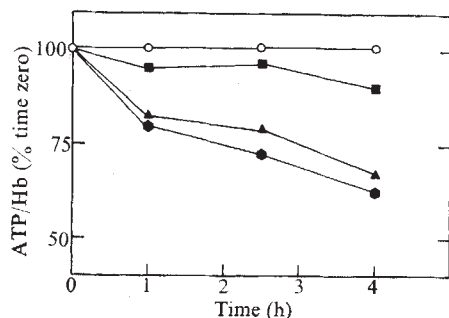


Fig. 3 ATP/Hb molar ratio in fish red blood cells incubated in media at pH 7.5 with no inhibitor (○), 10^{-2} M NaF (■), 5×10^{-4} M iodoacetate (▲) or 10^{-4} M NaCN (●). Values are from duplicate determinations which did not vary by more than 5%.

constant (about 7 p.p.m.) at each temperature. These animals also decreased their erythrocyte ATP with increased temperature (group II, Table 1). Moreover, fish acclimatised to 10 °C, but in air saturated water (12.5 p.p.m.), showed the same red blood cell ATP levels as those maintained at 10 °C but with 7 p.p.m. oxygen (Table 1). These data demonstrate that the ATP response was elicited by increased temperature alone and was independent of dissolved oxygen levels in the range 7–12.5 p.p.m.

Table 1 Comparison of red blood cell ATP/Hb molar ratios in *F. heteroclitus* acclimatised to different temperatures

Temperature (°C)	Group I	Group II
10	$1.84 \pm .05$	$1.81 \pm .07$
22	$1.68 \pm .06$	$1.72 \pm .08$
30	$1.31 \pm .08$	$1.29 \pm .06$

In group I, dissolved oxygen values at the various temperatures were: 10 °C, 12.5 p.p.m.; 22 °C, 9.0 p.p.m.; and 30 °C, 7.5 p.p.m. The dissolved oxygen for group II was maintained at a constant value of 7.0 p.p.m. during the course of the acclimatisation (4 weeks). All values represent averages of 10–12 fish. Errors are reported as $\pm$ s.e.m.

As outlined earlier, there is a significant ATP response when isothermal hypoxia is induced (that is, below 3 p.p.m.). Under hypoxia, *F. heteroclitus* also increased the number of circulating red blood cells (haematocrit) by 40%. This phenomenon has been reported and discussed previously for other fish species⁸. In an earlier study, we reported a significant increase in the haematocrit of warm-acclimatised *F. heteroclitus*¹¹. In the present study, however, we found no difference in haematocrit between fish acclimatised to different temperatures. In addition, red blood cell ATP concentrations of 30 °C-acclimatised fish (see Table 1) were higher than those described previously¹¹. To simulate natural conditions, oxygen levels were not controlled in our earlier study. Therefore, biological oxygen demand (microbial activity, and so on) at the higher temperature probably lowered dissolved oxygen into the hypoxic range. In these conditions, which *F. heteroclitus* often encounter in their natural habitat, temperature and oxygen act in tandem to control red blood cell organic phosphate and, in turn, Hb-O₂ affinity. When governed solely by its physical dependence on temperature, however, dissolved oxygen never reaches the low values required to induce hypoxia.

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Thyroidal trophic influence on skeletal muscle myosin

CROSS-INNERVATION experiments have convincingly demonstrated the importance of the nerve in determining the differential properties of skeletal muscle myosin. The role of hormones in deciding the phenotypic character of muscle myosin has received considerably less attention than that of the neural factors. Recent studies have shown that short-term alterations in thyroid status results in significant changes in cardiac muscle myosin^{1–4}. In contrast, some of those investigations also used skeletal muscle, but no apparent effects on skeletal muscle myosin were detected^{2,4}. But the greater half-life of skeletal muscle myosin would require a longer time than cardiac myosin to manifest these changes⁵. We have studied the effects of longer term hypothyroidism and hyperthyroidism on skeletal muscle myosin. We report here that alterations in thyroid status influence the quality of skeletal muscle myosin. Our observations suggest possible thyroidal involvement, along with or through neural trophic factors, in determining the phenotypic properties of skeletal muscle myosin.

Normal, hypothyroid and hyperthyroid male Sprague-Dawley albino rats with initial body weights of 180–200 g were subjects. Thyroidectomised animals were purchased from Hormone Assay (Chicago). Hyperthyroidism was induced in thyroidectomised rats by subcutaneous injections of 30 µg per 100 g body weight of Na 3,3',5 triiodo-L-thyronine on alternate days for six weeks. Animals were killed by exsanguination under pentobarbital anaesthesia (60 mg per kg). Thyroid status of the animals was confirmed by the enzyme activities of α -glycerolphosphate dehydrogenase (EC 1.1.99.5)⁶ and succinate dehydrogenase (EC 1.3.99.1)⁷ from whole homogenates of liver and soleus muscle at 25 °C (Table 1).

Immediately after excision a mid-portion of the soleus muscle was prepared for histochemistry. The tissue was mounted and frozen in isopentane cooled with liquid nitrogen. Cross-sections of 10-µm thickness were cut at –20 °C and stained for myofibrillar adenosine triphosphatase (M-ATPase). The staining procedure involved 10 min pre-incubation (37 °C) in glycine or 2-amino-2-methyl-1-propanol buffer (pH 10.30) containing 18 mM CaCl₂ followed by 30 min incubation in pre-incubation medium containing 3.08 mM ATP and adjusted to pH 9.40. This is essentially the method of Padykula and Herman⁸ as modified by Guth and Samaha⁹. The difference in staining intensity is based on the alkali stability and lability of fast and slow skeletal muscle myosin ATPase activity, respectively^{9,10}, resulting in a more intense stain for fast than slow muscle fibres at an alkaline pH.

Soleus muscles used for myosin isolation were pooled into groups of four and stored in glycerol at –20 °C. Myosin was prepared using the method of Bárány and Close¹¹. The yield of myosin protein was similar for each experimental group (Table 1). Mg²⁺-activated ATPase activity indicated minimal actin contamination (1.06 nmol per mg protein per min). Ca²⁺-stimulated myosin ATPase activity was determined at 37 °C at various pH values using the reaction mixture of Bárány and Close¹¹ and the inorganic phosphate assay of Rockstein and Herron¹². Protein determinations were by the Lowry method with bovine albumin as the standard¹³.

Histochemical observations indicate that thyroid status

Table 1 Effects of thyroid status on soleus muscle myosin

	Euthyroid	Hypothyroid	Hyperthyroid
Final body weight (g)	385 ± 6 (14)	297 ± 6 (14)*	242 ± 8 (8)*
Liver			
SDH activity	24.86 ± 0.79 (11)	18.07 ± 0.84 (11)*	36.10 ± 3.29 (7)*
GPDH activity	4.65 ± 0.18 (11)	3.18 ± 0.10 (12)*	11.80 ± 0.58 (8)*
Protein (mg g ⁻¹)	216 ± 9 (14)	210 ± 10 (13)	226 ± 9 (8)
Soleus muscle			
Wet weight (mg)	137 ± 3 (28)	120 ± 4 (28)†	119 ± 2 (16)†
SDH activity	3.07 ± 0.08 (18)	1.46 ± 0.03 (14)*	4.70 ± 0.07 (12)*
GPDH activity	1.47 ± 0.04 (18)	1.11 ± 0.02 (14)*	2.58 ± 0.09 (12)*
Protein (mg g ⁻¹)	185 ± 10 (15)	188 ± 8 (13)	186 ± 7 (14)
Myosin yield (mg g ⁻¹)	31.99 ± 3.84 (7)	34.36 ± 2.75 (7)	33.16 ± 2.94 (4)
Alkali-stable fibres (%)	15.95 ± 1.40 (8)	0.12 ± 0.07 (9)*	35.09 ± 2.03 (6)*

Values are means ± s.e.m. The number in parenthesis is the number of analyses. SDH, succinate dehydrogenase. GPDH, α -glycerolphosphate dehydrogenase. Enzyme activities are in μ mol per g wet wt per min.

*Statistically significant at $P < 0.001$.

†Statistically significant at $P < 0.01$.

can alter the proportion of alkali stable (fast) and labile (slow) M-ATPase fibres in rat skeletal muscle (Fig. 1). The soleus muscles from the hypothyroid animals consisted of <1% alkali-stable fibres as compared to 16% for the euthyroid muscles (Table 1). Whereas the hyperthyroid muscles were comprised of more than twice the percentage of alkali-stable fibres observed in the euthyroid muscles. Muscles of each group contained fibres with an immediately intense M-ATPase stain. These fibres were most prevalent in the hyperthyroid muscles. They were classified as alkali-stable fibres in the present study.

The pH profiles of myosin ATPase activity are shown in

Fig. 1 Transverse sections from the soleus muscle of *a*, euthyroid *b*, hypothyroid and *c*, hyperthyroid rats stained for myofibrillar ATPase ($\times 22$).

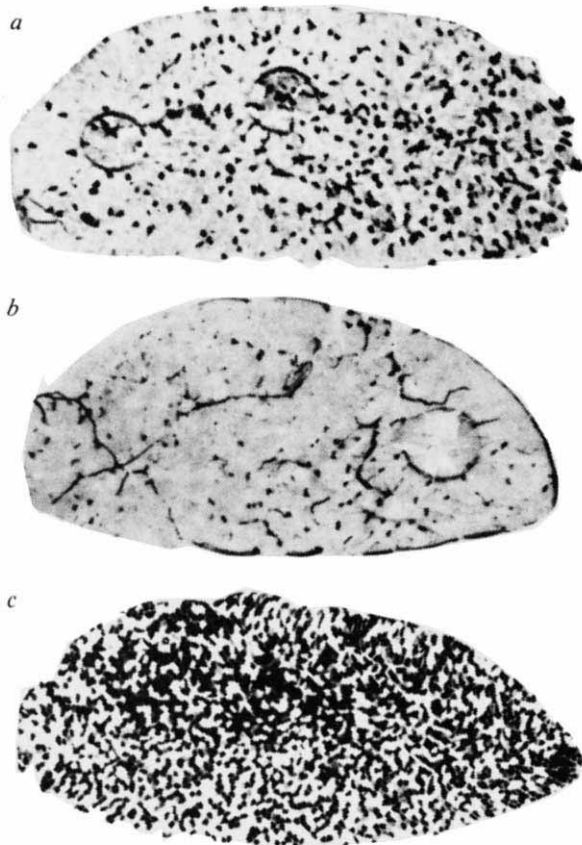


Fig. 2. The greatest differential effect on enzyme activity between the experimental groups was observed at pH 10.30. At this alkaline pH the euthyroid soleus exhibited only 54% of its activity at pH 7.40 as compared to 12% for the hypothyroid muscles and 236% for the hyperthyroid muscles. When comparing the activities at pH 10.30, the hypothyroid muscles had only 27% the activity of the euthyroid muscles. Whereas the hyperthyroid had a fivefold greater activity than the euthyroid muscles and 20-fold more activity than the soleus muscles of the hypothyroid animals at that alkaline pH.

As in the histochemical procedure, the basis for resolving fast-twitch and slow-twitch myosin ATPase is its property of being alkali resistant and susceptible, respectively¹⁰. The change in the alkaline stability of myosin isolated from hypothyroid and hyperthyroid muscles clearly shows that this property of the myosin protein has been transformed and that the transformation occurs in both directions, depending on the thyroid state. These biochemical findings, besides quantifying the changes in ATPase activity, have confirmed the histochemical observations for these experimental conditions. This is important since in certain conditions the histochemical method of staining for M-ATPase can be misleading^{14,15}. Further evidence for the validity of the M-ATPase stain was a complete reversal of the staining intensity of fast and slow fibres at pH 4.35, as occurs in normal muscle. The close agreement between the percentage increase in alkali-stable fibres (19%) and myosin ATPase activity (39%) at pH 7.40, considering fast-twitch myosin has three times the specific catalytic activity of slow-twitch myosin, imparts further credibility to the M-ATPase stain. This comparison, however, is not as highly correlated for the hypothyroid muscle.

It is well documented that fast-twitch and slow-twitch muscle myosin are qualitatively distinct proteins^{10,11,16}. It is widely accepted that these differences in myosin are primarily regulated by neural factors^{9,11,16,17}. Our results indicate that the thyroid state of an animal also has the potential for determining the phenotype of skeletal muscle myosin. The extent of the changes in myosin ATPase activity in soleus muscles which had been hyperthyroid for only six weeks was similar to that of changes observed in soleus muscle following long-term (6–15 months) cross-innervation with a fast nerve^{11,16,17}. Bárány and Close¹¹ observed a 50% increase in soleus myosin ATPase at pH 7.40 following 10–15 months of cross-innervation with a nerve normally innervating a fast-twitch muscle, as compared to a 39% increase for the hyperthyroid soleus. At pH 10.0, cross-innervated soleus muscle had a 125% increase in ATPase activity as compared to 70% for the hyperthyroid soleus at

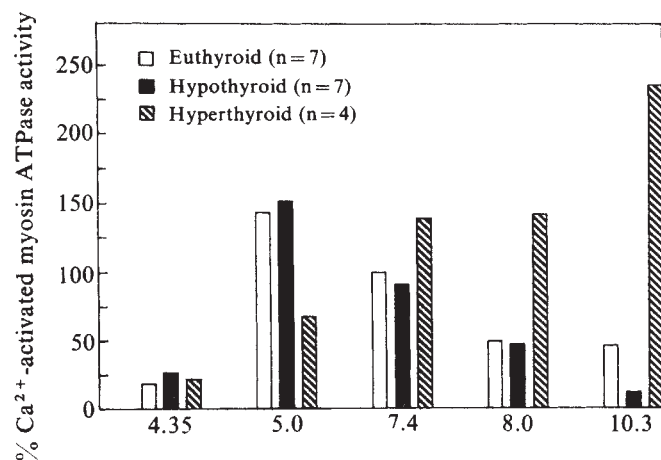


Fig. 2 % of Ca^{2+} -activated myosin ATPase activity relative to 100% for the euthyroid muscle at pH 7.40. The euthyroid soleus muscle at pH 7.40 at 37 °C had an activity of 0.18 μmol per mg myosin protein per min.

pH 10.30. In other studies^{16,17} the soleus exhibited increases of 35–75% in myosin ATPase (pH 7.8–8.0) 6–12 months following cross-innervation, whereas the hyperthyroid soleus had 186% greater activity than the euthyroid at pH 8.00. Furthermore, the extent of this transformation is probably not fully manifest in this six-week time period in the hyperthyroid muscle but it is probably nearly complete in the long-term cross-innervation studies. Longer term studies are in progress to allow for the changes to become more fully established, along with the added purpose of further categorising the changes in the myosin protein.

The findings of this investigation offer little insight into the mechanism by which the thyroid state alters the character of skeletal muscle myosin. It is possible the thyroidal influence directly and selectively stimulated muscle gene expression, affected neural trophic regulation, or produced other hormonal imbalances which induced this transformation.

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Hydrolysis of polyesters by lipases

INCREASING public concern about the treatment of waste materials has stimulated the study of the biodegradation of synthetic polymers. Among synthetic polymers, aliphatic polyesters are generally known to be susceptible to biological attack^{1–5}, but there are few reports of enzymes involved in their degradation. Bell *et al.*⁶ recently showed that the molecular weight of polycaprolactone (PCL) decreases on exposure to the acid protease from *Rhizopus chinensis* for 6–10 d (decreasing from 13,000 to 10,000). In addition, Tabushi *et al.*⁷ have found that polyesters composed of phenyllactic acid and lactic acid are hydrolysed by α -chymotrypsin. We showed previously that a polyester-degrading enzyme from *Penicillium* sp. strain 14-3, purified to a homogeneous state as exhibited by ultracentrifugal analysis and polyacrylamide gel electrophoresis, has properties resembling lipase⁸. It was not previously recognised that lipase acts on polyesters. We report here that several commercial lipases and an esterase also hydrolyse polyesters, and that *Rh. delemar* lipase is capable of hydrolysing various kinds of polyesters.

PCL and polypropiolactone were prepared by ring opening polymerisation of ϵ -caprolactone⁹ and β -propiolactone respectively in benzene in a nitrogen atmosphere at 60 °C with a diethylzinc–water catalyst system. Poly- β -methylpropiolactone (poly- β -hydroxybutyrate) was made from β -methylpropiolactone by the method of Yamashita *et al.*¹⁰ with a triethylaluminium–water catalyst system. Other saturated aliphatic polyesters were synthesised by a melt polycondensation technique¹¹, and unsaturated polyesters were synthesised by high temperature solution polycondensation¹². All alicyclic and aromatic polyesters were from Nihon Chromat except poly (tetramethylene terephthalate), from Union Carbide. The number average molecular weight (M_n) was measured by the vapour pressure equilibrium method with a Hitachi 117 apparatus.

Fig. 1 Gas chromatograms of the hydrolysis products of PEA by *Rh. delemar* lipase. PEA powder was filtered from the reaction mixture which was then analysed (a). The concentrated and dried reaction mixture was then esterified with diazomethane in ether (b). The hydrolysate of (a) with alkali was also analysed (c). Chromatographic conditions: Shimadzu GC-5A (flame ionisation) with glass column (1 m $\times$ 0.3 cm internal diameter) packed with Tenax GC. Temperature programmed at 5° per min from 135° to 345 °C (dashed line). Flow rate of both nitrogen and hydrogen was 50 ml per min. Arrows indicate ethylene glycol (EG) and adipic acid (AA).

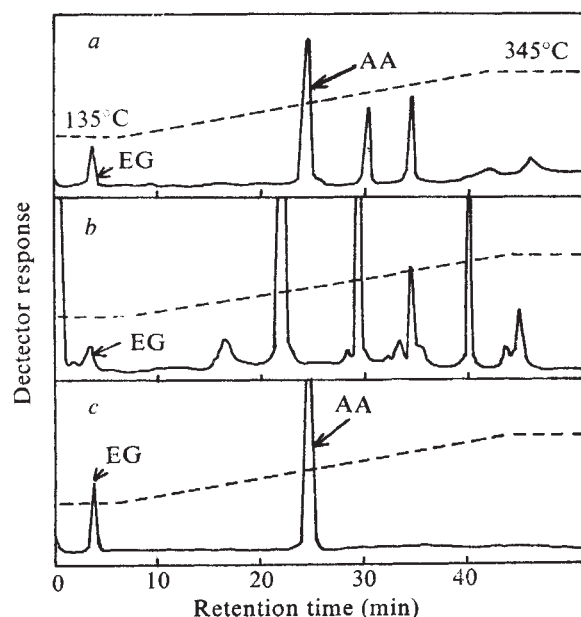


Table 1 Hydrolysis of polyesters by lipases

		TOC concentration (p.p.m.)			
Enzyme control		Formed by hydrolysis of polyester			
		Poly (ethylene adipate)	Poly- caprolactone	Poly(cyclohexylene- dimethyl succinate)	Poly(2,2-dimethyl- trimethylene iso- phthalate)
<i>Achromobacter</i> sp.	10	880	110	60	0
<i>Candida cylindracea</i>	5	3900	70	0	0
<i>Geotrichum candidum</i>	10	140	0	0	0
<i>Rhizopus arrhizus</i>	10	7400	60	40	0
<i>Rhizopus delemar</i>	5	6100	110	70	0
Hog liver (esterase)	30	940	90	120	0
(Substrate control)	—	(330)	(150)	(70)	(20)

Each reaction mixture contained 400 μ mol of phosphate buffer (pH 7.0), 300 mg of the polyester powder (particle size less than about 1 mm) and enzyme in a total volume of 10 ml. The enzyme concentration in each reaction mixture was adjusted to a degree of hydrolytic activity capable of liberating 420 μ mol of fatty acid from olive oil per 60 min at 37 °C at pH 7.0 (see ref. 8). Reaction mixtures were incubated on a rotary shaker at 180 r.p.m. at 30 °C for 16 h. After incubation, the TOC concentration was measured. No effect of the interaction between each substrate and inactivated enzyme preparation was observed. For example, when *G. candidum*, *Rh. arrhizus* or *Rh. delemar* lipase which was treated with a boiling water for 10 min, was allowed to react with PEA (PCL), the TOC concentrations in the filtrate of the reaction mixtures were 340 (150), 340 (160) and 330 (150) p.p.m. respectively as compared with 330 (150) p.p.m. of substrate control. The effect could not be examined in hog liver esterase because it was insolubilised by the heat treatment.

Achromobacter sp. and *Candida cylindracea* lipases (Meito Sangyo) were purified by gel filtration on Sephadex G-100 (2.6 $\times$ 79 cm) from crude preparations. Ultracentrifugally homogeneous preparations of *Geotrichum candidum* and *Rh. delemar* lipases were from Seikagaku Kogyo and partially purified preparations of *Rh. arrhizus* lipase and hog liver esterase were from Boehringer Mannheim Yamanouchi.

Hydrolysis of polyesters was measured by the rate of their solubilisation, and the measurement process does not necessarily involve complete hydrolysis into the constituent parts. The rate was determined by measuring the water-soluble total organic carbon (TOC) concentration at 30 °C in the reaction mixture using a Toshiba-Beckman TOC analyser. The precise reaction rates of polyester hydrolysis cannot be determined by this method, however, since the polyester powder particles in the reaction mixture vary in size and the solubility of the oligomers

formed by polyester hydrolysis differs depending on the kind of polyester. In the substrate and enzyme controls, enzyme or substrate was omitted from the reaction mixture.

Aliphatic polyesters, poly(ethylene adipate) (PEA) and PCL were hydrolysed by lipases from various microorganisms and hog liver esterase with the results shown in Table 1. *Rh. arrhizus* and *Rh. delemar* lipases showed especially strong activity. The action of hog liver esterase on PEA produced much more adipic acid and ethylene glycol, constituents of PEA, than did lipases. This suggests that the biodegradable property of aliphatic polyester comes from its susceptibility to hydrolysis by lipases or esterases. An alicyclic polyester, poly(cyclohexylenedimethyl succinate), was also hydrolysed by lipases from *Achromobacter* sp., *Rh. arrhizus* and *Rh. delemar* and by hog liver esterase. Of these, hog liver esterase showed the strongest activity. In contrast, an aromatic polyester, poly (2,2-dimethyl-

Table 2 Hydrolysis of polyesters by *Rh. delemar* lipase

Polyester	Mn	Powder size	TOC concentration (p.p.m.)	
			Substrate control	Formed by lipase
Poly(ethylene adipate)	2,720	C	330	8,360
Poly(ethylene suberate)	4,050	B	250	1,020
Poly(ethylene azelate)	4,510	B	570	3,080
Poly(ethylene sebacate)	1,570	A	390	550
Poly(ethylene decamethylate)	1,610	B	100	180
Poly(tetramethylene succinate)	4,240	A	270	150
Poly(tetramethylene adipate)	1,790	B	520	3,360
Poly(tetramethylene sebacate)	2,440	A	130	980
Poly(hexamethylene sebacate)	5,820	B	40	380
Poly(2,2-dimethyltrimethylene succinate)	2,370	A	360	240
Polypropiolactone	4,270	A	580	2,240
Poly-DL- β -methylpropiolactone	8,190	E	250	10
Polycaprolactone	6,740	A	150	310
Poly(<i>cis</i> -2-butene adipate)	2,700	C	1,270	580
Poly(<i>cis</i> -2-butene sebacate)	6,190	B	130	300
Poly(<i>trans</i> -2-butene sebacate)	3,560	A	910	340
Poly(2-butyne sebacate)	4,930	C	210	670
Poly(hexamethylene fumarate)		A	60	35
Poly(<i>cis</i> -2-butene fumarate)		B	390	30
Poly(tetramethylcyclobutane succinate)	3,440	E	10	0
Poly(cyclohexylenedimethyl succinate)	3,910	B	70	130
Poly(cyclohexylenedimethyl adipate)	3,250	B	80	200
Poly(tetramethylene terephthalate)		C	0	0
Poly(ethylene tetrachlorophthalate)	1,670	A	0	0
Poly(2,2-dimethyltrimethylene isophthalate)		A	20	0

All polyesters but two were prepared into powders at 30 °C by grinding. The exceptions were poly(tetramethylene terephthalate) which was powdered with a centrifugal ball mill and polycaprolactone which was powdered from a solution in chloroform by a precipitation method. The particle size of each polyester powder was ranked A, B, C, D or E, corresponding respectively to roughly less than 0.25 mm, less than 0.50 mm, less than 1.0 mm, 0.25–1.5 mm, 0.25–3 mm. Each reaction mixture contained 400 μ mol of phosphate buffer (pH 6.0), 300 mg of the polyester powder and 0.3 mg of *Rh. delemar* lipase in a total volume of 10.0 ml. The reaction mixtures were incubated in the same way as Table 1. The TOC concentration of the enzyme control was 10 p.p.m.

trimethylene isophthalate), was not hydrolysed by any of the enzyme preparations.

Rh. delemar lipase purified to homogeneity in the ultracentrifuge was found capable of hydrolysing various kinds of polyesters (Table 2). PEA was the best substrate tested, and activity was relatively high with poly(ethylene azelate). Polypropiolactone and PCL were degraded, but not poly(Δ^5 -methylpropiolactone), the Δ -isomer of which is known to be one of the materials stored by bacteria and algae. Unsaturated polyesters from 2-butenediol were also degraded, but polyesters with even a few hybrid bonds among the polymer chains, such as those induced from poly(hexamethylene fumarate) and poly(*cis*-2-butene fumarate), were hardly degraded. Two alicyclic polyesters, poly(cyclohexylenedimethyl succinate) and poly(cyclohexylenedimethyl adipate), were degraded fairly extensively. Aromatic polyesters such as poly(tetramethylene terephthalate), poly(ethylene tetrachlorophthalate) and poly(2,2-dimethyltrimethylene isophthalate) were not degraded.

Amongst the products of PEA hydrolysis by *Rh. delemar* lipase, we detected not only the constituent parts of PEA—adipic acid and ethylene glycol—but also large quantities of PEA oligomers (Fig. 1). This may indicate that *Rh. delemar* lipase randomly splits ester bonds.

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Involvement of DNA gyrase in bacteriophage T7 DNA replication

NOVOBIOCIN and the related antibiotic coumermycin A₁ (referred to as coumermycin) inhibit the supercoiling of circular double-stranded DNA catalysed by *Escherichia coli* DNA gyrase¹. They also inhibit the replication of chromosomal DNA in *E. coli* cells², and of circular double-stranded DNA in cell-free systems from *E. coli*^{3–6}. The activity of DNA gyrase obtained from a coumermycin-resistant (*cou*^r) mutant and the replication of colicin E1 plasmid (*Col E1*) DNA in a cell extract of the mutant are resistant to both drugs⁷. These results show that DNA gyrase is an essential component of these systems for the replication of the circular double-stranded DNA. It is interesting to examine whether DNA gyrase also has a role in replication of linear double-stranded DNA. The DNA of *E. coli* bacteriophage T7 is linear and double stranded⁸. Linear replicating molecules have been isolated from infected cells and it has been claimed that the phage DNA replicates as a linear form, at least in the first round of replication⁹. We show here that the replication of phage T7 DNA, including the first round, is inhibited by coumermycin.

The effect of various concentrations of coumermycin on DNA synthesis in coumermycin-sensitive and resistant bacteria during 30 min after infection of phage T7 was examined. T7 DNA synthesis was inhibited by coumermycin, to a greater extent in the sensitive bacteria than in the resistant bacteria (Fig. 1). To attain a similar extent of

inhibition, an approximately 10 times higher concentration of the drug was needed for the resistant bacteria than for the sensitive bacteria. At a given concentration of the drug, T7 DNA synthesis was inhibited to about the same extent as chromosomal DNA synthesis in the uninfected cells. This was true of both the sensitive and the resistant bacteria. On the other hand, it has been shown that a 50-fold lower concentration of the drug is sufficient to produce comparable inhibition in toluene-treated cells (refs 4, 13 and unpublished results). Thus the T7-infected cells seem to retain the same limited permeability to the drug as the uninfected cells. Phage DNA synthesis (Fig. 1) and chromosomal DNA synthesis (data not shown) in the resistant bacteria were inhibited by high concentrations of coumermycin. It is known that high concentrations (25 $\mu\text{g ml}^{-1}$ or higher) of coumermycin significantly inhibit DNA gyrase purified from the resistant bacteria (M. Gellert, personal communication) as well as other enzymes, such as DNA polymerase III and DNA-dependent RNA polymerase of *E. coli*⁴. The resistant strain used in this experiment was selected as a spontaneous drug-resistant mutant and its mutation was mapped close to the *dnaA* locus by P1-mediated transduction. Moreover, when *bglC*⁺ (the *bgl* locus is known to be located near the *dnaA* locus¹⁴) transductants of the resistant strain were selected, about 20% of them were found to be simultaneously coumermycin-sensitive, and two representative drug-sensitive transductants showed a drug-sensitivity of T7 growth similar to that of the

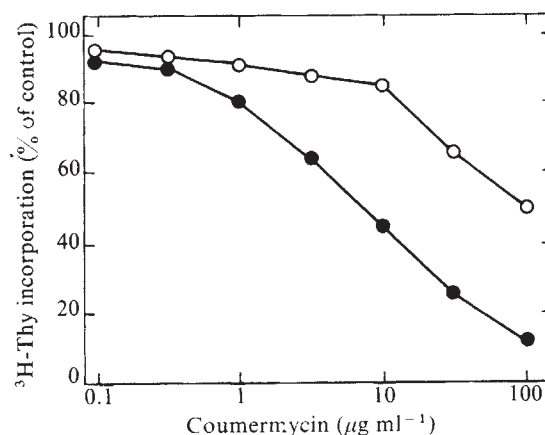


Fig. 1 Effect of coumermycin on DNA synthesis in T7-infected cells. NI746 (●), a low thymine-requiring mutant of NT525 *Su⁻bglCcou^s* and its spontaneous coumermycin-resistant (*cou^r*) derivative, NI751 (○) were used. The *cou^r* mutation of NI751 was mapped close to the *dnaA* locus. DNA synthesis in toluene-treated cells of NI751 and *Col E1* DNA replication in an extract of this strain were resistant to coumermycin. Cells were grown to 3×10^8 per ml in OC medium (a minimal medium with casamino acids (Difco))¹⁰ supplemented with thymine 2 $\mu\text{g ml}^{-1}$ at 37 °C, centrifuged and suspended in one-tenth volume of the absorption buffer (10mM Tris-HCl, pH7, 1mM MgCl₂, 0.5% NaCl, 10 $\mu\text{g ml}^{-1}$ gelatin, 0.4% casamino acids) with various concentrations of coumermycin (from J. Davies, University of Wisconsin). Coumermycin was dissolved in dimethyl sulphoxide and an equal amount of the solvent was added to the control cultures. Bacteria were infected with phage T7 at a multiplicity of five by incubating for 7 min at 30 °C. The adsorption mixtures were diluted 10-fold in OC medium with ³H-thymine (1.2 Ci mmol⁻¹, NEN) and various concentrations of the drug. After incubating at 30 °C for 30 min, acid-insoluble radioactivity in 0.1 ml of each culture was determined¹⁰—100% corresponds to about 12,000 c.p.m. A DNA–DNA hybridisation experiment¹¹ was performed with the samples made from the infected sensitive and resistant bacteria incubated with or without 100 $\mu\text{g ml}^{-1}$ coumermycin. In the sample made from the sensitive bacteria without the drug and those from the resistant bacteria with or without the drug, more than 80% of the labelled material was phage T7 DNA, whereas 75% was bacterial DNA in the sample made from the sensitive bacteria with the drug (data not shown). It is known that bacterial DNA synthesis does not stop immediately by T7-infection¹², and it is also known that bacterial DNA synthesis does not stop immediately after addition of coumermycin⁴.

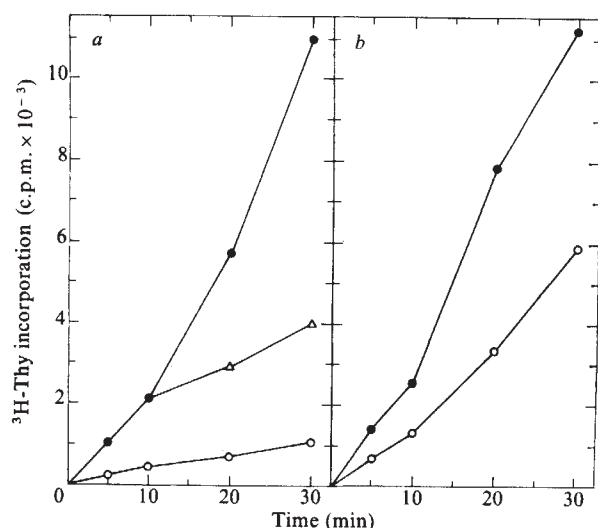


Fig. 2 Kinetics of DNA synthesis by T7-infected cells of NI746-*cou*⁺ (a) and NI751-*cou*⁺ (b). Methods used were generally as described in Fig. 1. Cells were grown in OC medium supplemented with thymine ($2 \mu\text{g ml}^{-1}$), centrifuged and suspended in the adsorption buffer with (○) or without (●) coumermycin ($100 \mu\text{g ml}^{-1}$). The cells were then infected with phage T7 at a multiplicity of five. After incubation for 7 min at 30°C , mixtures were diluted in the same medium with ^3H -thymine (1.2 Ci mmol^{-1}) and incubated at 30°C . After incubation for 10 min, the culture of the infected sensitive bacteria without the drug was divided into two portions, to one of which coumermycin ($100 \mu\text{g ml}^{-1}$) was added and incubation was continued (△). At intervals aliquots (0.1 ml) were withdrawn and acid-insoluble radioactivity was determined.

sensitive strain used in this experiment. Therefore, the possibility that the observed resistance of T7 growth to the drug was due to some unidentified drug-resistant mutant enzyme which might be involved in T7 growth should be very scarce. The differential effect of the drug on T7 DNA synthesis in the sensitive and the resistant bacteria indicates that the inhibition of the activity of DNA gyrase by coumermycin caused the inhibition of T7 DNA synthesis in the sensitive bacteria.

DNA gyrase could act directly on T7 DNA molecules, or it could act on the bacterial chromosome to produce and maintain its negatively supercoiled structure, which might be necessary for T7 DNA replication. When coumermycin $100 \mu\text{g ml}^{-1}$ was added at the beginning of infection, DNA synthesis in the infected sensitive bacteria was severely inhibited (Fig. 2a), whereas that in the resistant bacteria was relatively less inhibited (Fig. 2b). A similar inhibition of DNA synthesis in the infected sensitive bacteria was observed when the drug was added 10 min after infection (Fig. 2a). Since the bacterial chromosomes are extensively broken down by that time¹⁵, the inhibition of T7 DNA synthesis is not likely to be an indirect effect due to action of the drug on the bacterial chromosomes. This in turn shows that DNA gyrase acts on T7 DNA molecules.

To determine whether even the first round of replication of T7 DNA molecules is inhibited by coumermycin, the fate of the parental DNA after infection was examined. In this experiment, T7am233 (gene 6) was used with a multiplicity of infection of less than one to eliminate possible complexities in the analysis due to recombination and concatemer formation. It is known that neither recombination nor formation of concatemers of T7 DNA occurs in the absence of a functional gene 6 product, although several rounds of semiconservative DNA replication take place¹⁶⁻¹⁸. Both the sensitive and resistant bacteria incubated for 20 min in 5-bromodeoxyuridine (BrdUrd)-supplemented medium were infected with ^{32}P -labelled T7am233 and incubated for 12.5 min at 30°C with or without $100 \mu\text{g ml}^{-1}$

coumermycin. DNA was extracted and analysed by equilibrium CsCl density gradient centrifugation. For the DNA prepared from the infected sensitive bacteria incubated without the drug, most of the labelled DNA appeared at the position of fully light (LL) DNA, and about 25% formed a broad band with a peak approximately at the position of half-heavy (HL) DNA (Fig. 3a). The shoulder on the heavier side of HL DNA probably consists of replicative intermediates in the second or later round of replication. T7am233 DNA replication in each infected cell was quite heterogeneous. The DNA replication of T7am⁺ as well as a gene 6 amber mutant has been shown to be rather heterogeneous in the experiments similar to that shown here; a considerable portion of the parental DNA still forms a band at the position of LL DNA, even when the progeny DNA labelled with BrdUrd appears at the position of fully heavy (HH) DNA, and replicated DNA molecules form rather broad bands at the positions of HL and HH DNA^{16,17}. For the DNA prepared from the infected sensitive bacteria incubated with coumermycin, however, practically no labelled DNA appeared in the position of HL DNA (Fig. 3c). In contrast, when the resistant bacteria were used, the amount of labelled DNA

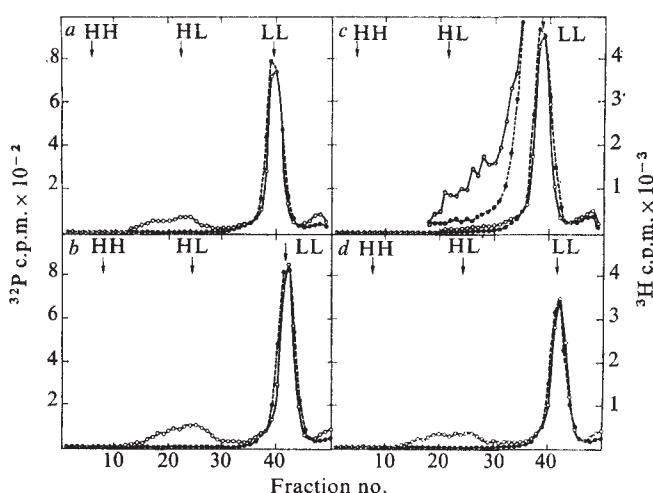


Fig. 3 CsCl density gradient centrifugation of DNA extracted from cells of NI746*Su-cou*⁺ (a and c) and NI751*Su-cou*⁺ (b and d) infected with ^{32}P -labelled T7am233. Cells were grown in OC medium supplemented with thymine ($2 \mu\text{g ml}^{-1}$), centrifuged and suspended in OC medium supplemented with BrdUrd ($5 \mu\text{g ml}^{-1}$, Sigma) in place of thymine and further incubated for 20 min at 37°C to replace the intracellular pool of nucleotides. Cells were then centrifuged, suspended in the adsorption buffer with (c and d) or without (a and b) coumermycin ($100 \mu\text{g ml}^{-1}$), and infected with ^{32}P -labelled (40 mCi mmol^{-1}) T7am233 (phage strain obtained from H. Ogawa, University of Osaka) at a multiplicity of 0.6. After incubation for 5 min at 30°C , the cultures were diluted in OC medium supplemented with BrdUrd and with or without the drug, and incubated at 30°C . At 12.5 min after the dilution, the reaction was stopped by addition of four volumes of cold SSC (0.15 M NaCl , $0.015 \text{ M Na}_2\text{ citrate}$)– 5 mM KCN . Cells were centrifuged and suspended in 0.2 ml of SSC (pH adjusted to 8.0)– 10 mM EDTA – 10 mM KCN . Lysozyme (Sigma) was added at a concentration of $500 \mu\text{g ml}^{-1}$ and the mixtures were frozen and thawed three times. After incubation for 15 min at 37°C , sodium *N*-lauroyl sarcosinate (Sigma) was added at a concentration of 0.1% and the mixtures were incubated for 15 min at 65°C . The cell lysates were then treated with proteinase K ($200 \mu\text{g ml}^{-1}$, E. Merck) at 37°C for 2 h. The lysates were diluted with SSC and after addition of ^3H -labelled (2.5×10^4 c.p.m.) *E. coli* DNA, brought to a final volume of 7.5 ml with a density of 1.73 g ml^{-1} by adding CsCl. They were centrifuged in a Beckman 40 rotor for 50 h at $36,000 \text{ r.p.m.}$ at about 15°C . Each gradient was collected from the bottom of the tube to give about 50 fractions, and ^{32}P (○) and ^3H (●) counts were determined. Approximately 80% of the input radioactivity was recovered from each gradient. Positions for light (LL), half-heavy (HL) and heavy (HH) DNA are indicated by the arrows. In c, the distribution of ^{32}P and ^3H counts in the fractions 18–35 is also shown on a 10-fold expanded scale.

which formed a band around the position of HL DNA was only slightly reduced by the addition of the drug (Fig. 3b, d). These results indicate that the inhibition of replication of T7am233 DNA molecules in the sensitive bacteria was due to inhibition of DNA gyrase by coumermycin.

The inhibition of the first round of replication could occur at initiation and/or at an early stage of DNA replication. In the preparation made from the infected sensitive bacteria incubated with the drug, a small but significant amount of labelled DNA was found between the positions of HL DNA and LL DNA (see Fig. 3c inset), even though practically no band of the labelled DNA was found in the positions of the HL or heavier DNA. These molecules are likely to be replicative intermediates arrested by the drug early in replication, rather than those on the way of the normal replication resulting from the incomplete inhibition by the drug of the initiation of DNA replication. This leads to the conclusion that DNA gyrase is involved at least in the stage of elongation of DNA chains.

Phage T7 DNA has redundant ends and could circularise through that structure¹⁹. Circular molecules have been only rarely observed in T7am⁺ DNA obtained from bacteria at an early stage of infection, however (refs 2, 9 and unpublished results of T. Ogawa and J.T.). Use of T7am233 reduced the possibility of circularisation of phage DNA molecules mediated by the product of gene 6. Because the DNA of T7am⁺ as well as a gene 6 amber mutant¹⁷ seems to replicate as a linear molecule, the inhibition of the first round of replication of T7am233 DNA molecules by coumermycin implies that DNA gyrase is involved in the replication of linear double-stranded DNA.

When a duplex DNA molecule replicates, the parental strands must unwind to serve as templates. If the replicating molecule is circular and covalently closed, at least a temporary nicking of one of the strands is essential for the unwinding. On the other hand, if the replicating molecule is linear, the unwinding could be accomplished by relative rotation of the replicated region in relation to the unreplicated region of the molecule without nicking of the strands. But, such rotation for unwinding of a long duplex DNA molecule *in vivo* is quite improbable, if one considers the necessity of simultaneous rotation of molecules attached to the DNA molecule, such as the machinery for transcription and translation and cellular structures like membranes²⁰. It has been suggested that phage T7 DNA in infected cells is associated with the bacterial membrane²¹⁻²³. The unwinding could be facilitated by nicking and closing of one strand which would occur at or a little ahead of the replicating point. A nicking and closing enzyme may act to relieve the positive supercoiling strain imposed by replication, or more actively, to facilitate separation of the parental strands by imposing a negative supercoiling strain. DNA gyrase would be a suitable enzyme to act in this process.

We thank M. Gellert for useful comments.

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Is the centriole bound to the nuclear membrane?

THE persistence of the centriole during interphase near the nucleus within the so-called cell centre seems to be a general feature of most animal cells. An association between the nucleus and the cell centre has been proposed and has been clearly shown in some cell types^{1,2}. A permanent system involving chromosomes, spindle fibres and centrioles has been suggested by Lettre *et al.*³, and Aronson⁴ has demonstrated the existence of a Colcemid-sensitive mobile system between the nucleus and a centre at the two-cell stage in the sea urchin *Lytechinus variegatus*. We show here that there is a physical association between the centriole and the nucleus in the rat liver cell which resists cold treatment, cell disruption and nuclear isolation. When nuclear envelopes are prepared from isolated nuclei, the association between the centriole and the nuclear envelope is maintained.

Nuclei from rat liver were prepared according to Blobel and Potter⁵ with slight modifications to adapt the procedure to larger amounts of tissue. Liver tissue (33 g) was homogenised in 110 ml of 2.2 M sucrose-TKM (50 mM Tris-HCl pH 7.5, 25 mM KCl, 5 mM MgCl₂) with a Chauveau type homogeniser (Plastimarine). The homogenate was filtered through 10 layers of cheese cloth and mixed with 220 ml of 2.2 M sucrose-TKM, using a glass rod. The suspension was transferred to cellulose nitrate tubes underlaid with 10 ml of 2.3 M sucrose-TKM and centrifuged for 1 h at 4 °C and 25,000 r.p.m. in an SW 25.2 rotor (Beckman). The nuclei were then washed by resuspension with 1 M sucrose-TKM and with 0.25 sucrose-TKM.

These nuclear preparations were pure as judged by electron microscopy. Nuclei showed intranuclear structures such as nucleoli and perinuclear and perinucleolar chromatin. The nuclear envelope was present, contained pores and had ribosomes associated with the outer leaflet. The chemical composition of these preparations was constant, suggesting good reproducibility of the isolation technique. The lipid phosphorus/DNA phosphorus ratio was 0.060 ± 0.002 (over 20 determinations). This value is low, indicating a low cytoplasmic contamination. Presence of centrioles in such nuclear preparations was not detected by electron microscopy (although we did not make a systematic search using serial sections).

Nuclear envelopes were isolated from such nuclei by the use of heparin which, in suitable conditions, induces complete solubilisation of chromatin^{6,7}. This allows the observation of well-preserved nuclear envelopes which have not been damaged by sonication, high salt or shearing through sucrose gradients. Nuclei were resuspended in 0.01 M Tris-HCl pH 8.0 containing 0.01 M Na₂HPO₄ at a final concentration of 200 µg DNA per ml. Heparin was then added in an excess corresponding to 1.5 times (w/w) the amount of DNA present in the nuclear suspension. After short vortex mixing the nuclear suspension was diluted five times with buffer and nuclear membranes were sedimented by centrifugation for 40 min at 20,000 r.p.m. (45,000g). All operations were performed at 4 °C.

Electron microscopic observation of such nuclear envelopes showed a surprisingly high number of centrioles. Some examples

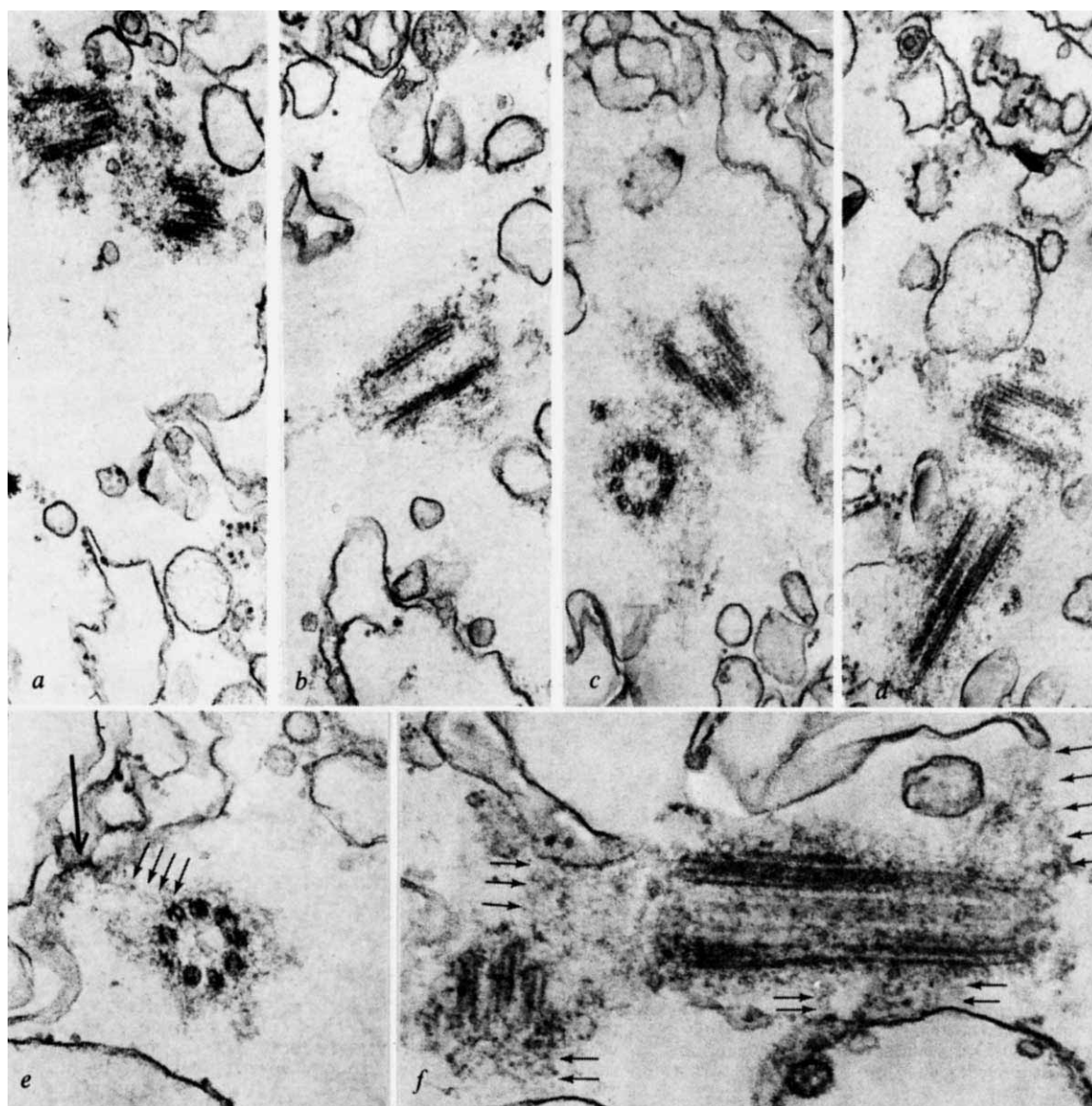


Fig. 1 Presence of centrioles in nuclear membrane pellets. Rat liver nuclei were resuspended at 4 °C in 0.01 M Tris-HCl pH 8.0 containing 0.01 M Na_2HPO_4 and incubated with heparin (Na^+ salt-grade I from Sigma) at a heparin to DNA ratio of 1.5 (w/w). After short vortexing, the nuclear suspension was diluted fivefold with buffer; nuclear membranes were sedimented immediately in that case at 45,000g for 40 min. Nuclear membrane pellets were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. They were postfixed in 2% osmium tetroxide, dehydrated in ethanol and embedded in Epon-Araldite. *a-d*, 42,000 $\times$; *e, f*, details showing apparent attachment (small arrows) of centrioles to membranes through dense material surrounding them. *e*, 60,000 $\times$; *f*, 72,000 $\times$.

are shown in Fig. 1. They sometimes seemed to be bound to the nuclear envelope by the surrounding material (Fig. 1*e-f*). We assumed that the failure to see centrioles in preparations of whole isolated nuclei was due to the relative size of centrioles compared to nuclei. Such a difficulty should not occur with optical microscopy which allows the observation of whole nuclei. Isolated nuclei, suspended in the isolation buffer (0.05 M Tris-HCl, pH 7.5, 0.025 M KCl, 0.005 M MgCl_2) showed irregular shapes, intranuclear organisation and nucleoli when observed by phase contrast microscopy. It remained difficult to detect the presence of centrioles in these nuclei.

The ability of low Mg^{2+} (5×10^{-4} M) to decondense heterochromatin^{8,9} was exploited to produce swollen and spherical nuclei with homogeneous nucleoplasm, in which all apparent organisation is lost except faint nucleoli. In such conditions, it was easy to detect centrioles associated with nuclei either by phase contrast microscopy or in polarised light. Several examples are given in Fig. 2. More than 50% of the nuclei in any pre-

paration had a centriole which could be recognised without ambiguity because of its typical aspect as two paired dots in phase contrast microscopy or polarised light microscopy. In rare circumstances, centrioles could be seen as two orthogonally oriented small bars (Fig. 2*f, j*); change in focus could show centrioles as black dots or refringent white dots (Fig. 2*e, i*). With some nuclei, by changing the focus, one could observe the centriole as two white dots, two black dots, a white and a black dot and the reverse situation (Fig. 2*e*). This is because of the complex spatial arrangement of centriole in two orthogonally oriented bars. In some cases varying the focus caused one or two other dense spots to appear on the same nucleus (Fig. 2*g, h, i*), but the meaning of this observation is not known.

In some nuclei, fibrillar structures could be seen originating in centriole. This would suggest the existence of an 'aster-like' system in the liver cell, and that part of it may remain associated with centriole during cell disruption and cold treatment.

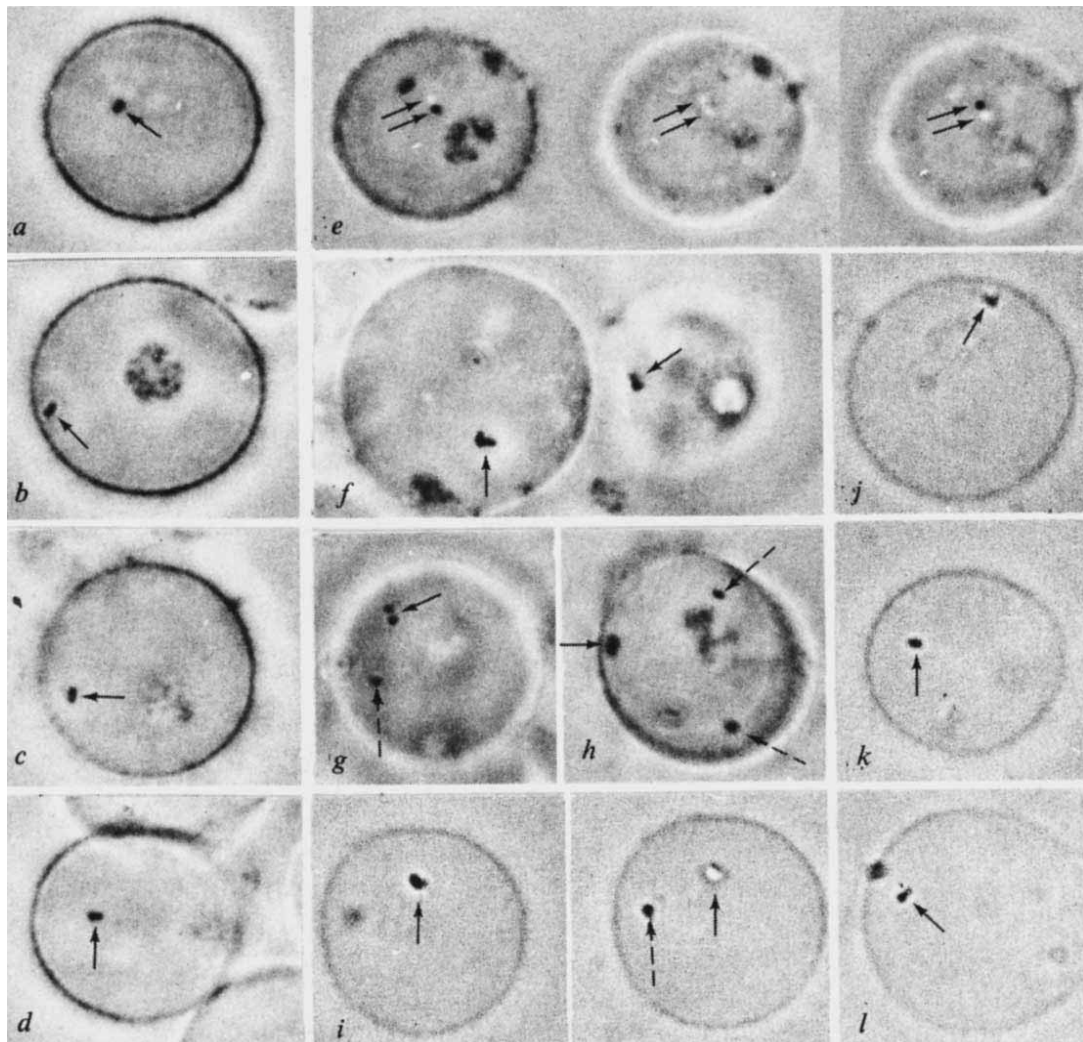


Fig. 2 Examples of rat liver nuclei resuspended at low magnesium concentration (0.05 M Tris-HCl pH 7.5, 0.025 M KCl, 5×10^{-4} M MgCl_2) which show associated centrioles. Magnification is $2,100 \times$. Arrows indicate centrioles. *a-h*, Phase contrast microscopy; *i-l*, polarised light; *e* and *i*, various focusings on the same nucleus which show that centrioles can appear as two dark dots, two refringent dots or one dark and one refringent dot. *f* and *j*, centrioles appearing as two orthogonally-oriented bars. *g* and *h*, nuclei showing other dark dots (dotted arrows) beside centrioles.

This association centriole-nucleus is probably not restricted to the liver cell, as it was also seen in preparations of thymocyte nuclear membranes.

What is the significance of these observations? A major role has been attributed to the cytoskeleton in the control of cellular functions such as cell shape, cell movements, cell polarity and cell surface activity. This raises the question of the exact role of the association of the microtubule organising centre with centrioles during interphase.

One role for such a binding between nucleus and centriole could be the anchorage of nucleus into the cell cytoplasm. The methods introduced by Prescott *et al.*^{10,11} to enucleate cells make use of cytochalasin B, and enucleated cytoplasms obtained in this way always contained centrioles¹²⁻¹⁴. One can therefore speculate that it is the disruption of the binding of nucleus to centriole which allows cell enucleation, although an alternative hypothesis has been proposed by Wright *et al.*¹⁵ which makes the subplasmalemmal network of microfilaments the target of the cytochalasin B effect. The effect of cytochalasin B suggests that the binding of the nucleus to the centriole is at least in part realised by actin filaments. That not only actin is involved is indicated by the effect of colchicine¹ and colcemid² on the centriole-nucleus complex.

The well-known biological effect of cytochalasin B on cell cultures is to impair cytokinesis but not karyokinesis, leading to bi- or multi-nucleated cells¹⁵. Thus, the structural role proposed

here for the nucleus-centriole complex, that is, anchorage of the nucleus into the cell, would indeed have a functional counterpart.

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reviews

Islands in space

R. L. F. Boyd

The High Frontier: Human Colonies in Space. By G. K. O'Neill. Pp. 288. (Jonathan Cape: London, 1977.) £5.95.

A POPULATION of several thousand million people located in space colonies, within a mere 35 years of the initiation of a space colonisation programme, is typical of the thinking—one might even say the evangel—of Dr O'Neill. In this readable and extraordinarily optimistic promotion of the cause of space as better than this Earth the author argues that man must be persuaded to rid himself of his "planetary chauvinism" and accept that even the Moon or Mars, for example, would not provide as suitable an environment as man-made habitats situated at one of the semi-stable Lagrangian points in the Earth-Moon system.

Three stages of development are suggested. "Island One" would be constructed primarily of aluminium and glass inside a sphere of 0.5 km diameter. The habitat, filled with an oxygen atmosphere, would rotate to provide an Earthlike gravity at the equator. The great majority of materials for the construction would be mined and transported from the lunar surface. Heavy industry, located nearby outside the sphere, and agriculture would, it is claimed, benefit from the continuous and predictable supply of solar energy, and the whole system could support a population of 10,000 in some comfort. Careful screening would prevent any unwanted "bugs" from being taken in from Earth; and presumably no malign mutation is expected to occur.

Within a further 50 years, the scale of development from these small beginnings is envisaged as leading to the "Island Three" habitats, cylinders 4 miles in diameter and 20 miles in length, maintaining several million inhabitants. There is probably no reason to dispute that such a design is already within the limits of present-day structural materials. Economic motivation for such colonies is claimed to lie with the construction of large solar power stations, subsequently placed into geosynchronous orbit and providing the Earth with electricity through high power microwave transmission. Such systems have, of course,

already been the subject of detailed design studies under NASA sponsorship, but it is not clear why sunlight in space should be more economic than in the desert areas of the near tropic latitudes here.

This book may properly rank as space fiction rather than prophecy but if so it is not because of any violation of scientific laws. No fundamentally new physics is required nor any great projection of present-day engineering practice. The speed with which such plans could be formulated and implemented even if adequate political backing was forthcoming, as suggested by O'Neill, is a point on which opinion will, however, vary greatly. In particular, there is little discussion of the inevitable sociological, legal and personal, including moral and religious, questions in their widest sense.

Much space is devoted to the projected economic aspects of space colonisation, particularly in terms of the initial investment, and to considering the viability of selling power to Earth through the solar power stations, considerations inevitably of a very speculative nature and, in themselves, hardly a complete justification for this mammoth scheme.

In chapter 2, entitled "The Human Prospect on Planet Earth", O'Neill presents what he sees as the most compelling arguments for a massive

colonisation of space. The shortage of land and energy on Earth are even now an obvious cause for concern and, with the expected rate of population growth, the situation may become desperate even before the middle of the next century. It may be thought, however, that these are basically political and ethical questions, that given good will, unselfishness, honesty, education, tolerance, sympathy and so on, it would be no more difficult to build a heaven on Earth than a "New Earth" (the title of chapter 4) in the heavens.

The New English Bible translates an aspiration of the early Christian church from two millennia ago, and no doubt echoing a far older yearning of many people, as "we . . . look forward to new heavens and a new Earth, the home of justice". This problem with its associated need for wisdom and moral fibre outweighs all the technical difficulties we face. Solve this and we might avoid the energy crisis of the late 1980s and the serious risk of war between "haves" and "have-nots" before the end of the century. □

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Animal cells in culture

Biochemical Methods in Cell Culture and Virology. By Robert J. Kuchler. Pp. ix+331. (Halsted/Wiley: New York and Chichester, UK, 1977.) £22.50; \$38.

BECAUSE research in animal virology is based to a large extent on the use of animal cells in culture, it might seem a good idea to include in one manual the procedures of both. In fact, the task is not an easy one, as this review will suggest.

The stated aim of this book is to provide a guide for growing, handling and studying viruses and cell cultures in the hope that the student considering a career in virology, cell biology or cancer research should find it useful. There are three parts, of about equal lengths: Cell Culture, Virology, and

Macromolecular Analysis. The content is essentially a collection of very many procedures. Each procedure, or group of related procedures, is preceded by a brief introduction on its purpose and, very cursorily, on its history.

Not many students can secure a copy of the standard procedures, formulations, recipes, and so on, which several large research laboratories, dealing with virology and cell cultures, necessarily keep. These procedures are often collected in loose-leaf Laboratory Manuals. Every time that a procedure or formulation is changed, it is an easy matter to replace the corresponding pages.

The next best method (though expensive) for a student who cannot get hold of any such manual, is a book; but a book does not have replaceable

loose pages. In the two fields of virology and cell culture the speed of advance of methods is very fast, much faster than that of ideas. Thus, a manual on methods requires continuous updating.

Even the short time between going to press and publication has had this undesirable effect. Two examples: the procedures for cell fusion stop at the (doubtfully practical) use of lysolecithin (1972); that is, before the introduction of polyethyleneglycol; and in the section on "Macromolecular Analysis", sequencing of viral DNA genomes by means of restriction endonucleases is not even mentioned.

This book thus falls between two stools. As a manual on methods it is, inevitably, already outdated in parts and will very soon become extensively so. As a general survey of viruses as probes for cell biology its aim is not

wide or high enough. Perhaps fewer methods, just as examples, but a much deeper and wider discussion on the current problems of virology and on how those methods contributed to solving them, would have made the book more stimulating. The student is much more helped by making him interested enough to look up the literature on procedures than by providing him with ready-made recipes of very short half-life. As it is, this book will be more valuable as a source of references in the libraries of research laboratories than as a laboratory manual at the bench, or as reading for postgraduate students.

G. Pontecorvo

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Mammalian fertilisation mechanisms

Fertilization Mechanisms in Man and Mammals. By Ralph B. L. Gwatkin. Pp.x+161. (Plenum: New York and London, 1977.) \$21.

FERTILIZATION is intrinsically a tremendously important process, marking as it does the union of male and female gametes, the recombination of their genetic loads, and the initiation of the new individual. Only in comparatively recent times, however, have various aspects of fertilisation in mammals become susceptible to direct experimentation. The earlier work was consequently restricted largely to non-mammalian forms, notably the marine invertebrates. This area of investigation has continued to be most productive and is now experiencing a recrudescence of research interest, which, together with studies based on the new capabilities in the handling of mammalian gametes, is yielding a wealth of new information. The time is ripe for an up-to-date and critical collation of published data from both mammalian and non-mammalian sources. Gwatkin's book goes a long way to meeting this challenge and should certainly fill a need.

Chapters in the book deal with the development and properties of the gametes, their transport in the female tract, fertilisation *in vitro*, sperm capacitation, the acrosome reaction, sperm penetration of the zona pellucida, sperm-egg fusion, the defence against polyspermy, pronucleus formation and the metabolic events detected during fertilisation. Additionally, atten-

tion is given briefly to the fate of surplus spermatozoa in the female tract and their interaction with somatic cells, and to the results of recent work on parthogenesis. There is also an Epilogue in which the author indicates directions for future research.

In the relevant chapters, particular attention is given to the exploration of the surface properties of gametes using lectins and other agents, and the close study of the successive stages of interaction of egg and spermatozoon which betoken different orders of response. A good deal of mystery still attaches to these processes and this in itself should serve as a powerful stimulus to further research. A fascinating feature of this phase of fertilisation lies in the apparent duplication of gamete properties and faculties, which denotes a curious limitation in the forms of substrate on which the influence of evolution has had to operate. The consolation for the biologist lies in the possibility of drawing useful inferences from what can be regarded as complementary patterns.

The book is written in a clear concise style and bears the authoritative stamp of an active and highly productive investigator in the field. The wide range of the author's acquaintance with the literature is evident throughout the text and in the extensive list of references provided.

The book can be warmly recommended to all those involved in research on gamete physiology and fertilisation, in non-mammals as well as mammals, and as an essential source of information and ideas for advanced students of medical and biological sciences.

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Logical foundations of computation

The Theory of Computer Science: A Programming Approach. By J. M. Brady. Pp. xiii+276. (Wiley: New York and London, 1977.) Hardback £8; paperback £4.50.

BRADY has written a book which can be warmly recommended to readers wishing to understand the logical foundations on which computation rests. It will be particularly valuable to students of computer science, but may be of interest also to computer programmers who wish to widen their horizons.

As a practical discipline, computing grew up in the late 1950s and early 1960s. Recruits to the programming profession came from mathematics, engineering, science, and even the classics and humanities. These people applied a largely intuitive approach to solving problems pressed upon them by commercial and other users, urgently requiring effective solutions. There were many difficulties, and many projects were late in completion, cost more

than was intended, or were even aborted before completion. The lessons learned during this period led to the formulation of a set of methodologies for program design and construction which are now often graced with the name "Software Engineering".

To aid the newly emerging discipline, theoretical foundations have been and are being developed. Methods of defining the syntax and semantics of programming languages, methods of proving the correctness and equivalence of programs, and of analysing the complexity of algorithms, are theoretical developments with immediate potential impact.

Arising, however, from the mathematics and logic camp there has come a theoretical background to computing which is often criticised as quite irrelevant to the practising programmer. Brady argues that this is due to a misunderstanding of its position in the logical structure of the subject. The theories of computability, based on Abstract (Turing) Machines and General Recursive Functions belong to the meta-theory the goals of which are to define precisely the meaning of 'computable' and to discover the theoretical limits of computability.

His book is accordingly divided into

two parts. Part 1 covers the meta-theory, whereas part 2 (which has a different title in the contents list and in the text) discusses the approach to a theory of computer science.

Brady does not assume extensive mathematical knowledge of his readers, and the prerequisites are summarised in an appendix. Throughout the book he leans heavily on the intuitions of the reader as an experienced programmer. He compares his approach with that of M. L. Minsky (*Computation: Finite and Infinite Machines*, Prentice-Hall 1967) by suggesting that his book is to programming what Minsky's is to hardware; hence the second part of his title. He writes with an enthusiastic style which makes his text very readable. There are frequent invitations to the reader to extend his understanding by proving lemmas and solving problems. These he calls "checkpoints", a usage which I find irritating. There are also more typographical errors than one expects in a book of this quality. These are, however, minor blemishes in an otherwise excellent book.

S. J. Goldsack

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Neutrino proceedings

Proceedings of the International Neutrino Conference, Aachen 1976. Edited by H. Faissner, H. Reithler and P. Zerwas. Pp. xii+748. (Vieweg: Braunschweig, 1977.) DM168.

It has been interesting to review this conference report while attending the 1977 Lepton-Photon Symposium in Hamburg, which covers the same field and much more. Rather than making last year's news seem old and dull, this year's conference illustrates how useful the Aachen proceedings can be as a reference. Aachen was a specialised conference and the speakers had time to do their experiments and theories justice. At conferences with a wider spread of subject matter the results can be reported but the details are lost.

Some of the results reported at Aachen have, of course, now been overtaken by better experiments, or modified by more careful analysis. Nevertheless, much of the picture is the same as it was last year. The four-quark theory, with charm, still stands and a great deal of the evidence for this is reported in these proceedings; including a few wisely chosen non-neutrino results from e^+e^- storage rings. The quark-parton picture of

hadrons is being increasingly refined, with about the correct amount of scale-breaking to satisfy the asymptotically free gauge theories. As well as data on these effects there is a useful review by the late Benjamin Lee, who includes a simple introduction to scale-breaking in the course of his final summary of the whole conference.

Theoretical contributions from Dalitz, Cabibbo, Salam and others give a picture of the variety of different quark models still available. M. K. Gaillard and de Rujula discuss the success of the charm concept in the light of the rich spectrum of particles reported in the experimental sessions. In contrast to their confidence, there are calls from Wolfenstein, from Fritsch and from Sakurai for more data on neutral currents, on second class weak currents, on neutrino oscillations, on parity violation in atomic physics, and so on. We certainly do not yet understand the weak interaction completely.

As well as high energy accelerator physics, there are a few papers on neutrino astrophysics and on assorted low-energy topics such as double β decay, and the ^3H spectrum as a probe of the neutrino mass.

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Prosimian behavioural ecology

Ecology and Behaviour of Nocturnal Primates. By P. Charles-Dominique. Pp. x+277 (Duckworth; London; Columbia University Press: New York, 1977.) £12.50; \$21.90.

THE behavioural ecology of the prosimians, an essential element in our understanding of the evolution of the order of Primates as a whole, raises special problems not encountered in field studies of monkeys and apes. The majority of prosimians are small, shy inhabitants of dense forest environments, solitary in their active phase and, most pertinent here, nocturnal. The task of adequately studying these in the wild is daunting and might seem impossible at times. Yet this book is a measure of just how successfully that task can be carried out.

In the main, it presents the results of almost a decade of research in Gabon in West Africa on five sympatric species of lorises (three bushbabies, the potto and the angwantibo) living in the equatorial rainforest. Covering in great detail such areas as feeding and diet, stratification, locomotion, defence against predators, and many aspects of social behaviour and demography, it is concerned not simply with each species viewed in isolation but with the vital questions arising from their sympatry. It succeeds in shedding considerable light on the evolution of this group, the adaptive strategies called forth by the particular and precise ecological niches they occupy, the specialisations of structure and behaviour that have developed, and especially the manner in which competition between the species has been minimised.

In most instances, penetrating and wide-ranging analysis leads to well-rounded and convincing synthesis. This has been achieved in two ways: first, by the skilful use of a variety of field techniques, ranging from direct observation with headlamp to capture-mark-release procedures and radio-tracking, the latter yielding some most valuable information. Second, the author repeatedly recognises or finds confirmation of his emergent hypotheses in acute 'casual' observations of untrammelled animals, or in beautifully simple manipulations of the forest environment or captive circumstances.

All this is not to say that Charles-Dominique's account of the Gabonese prosimians is complete or without fault. In places, a paucity of hard data, unrevealed sample sizes or the absence of detail when procedures are des-

cribed, raise doubts in the mind. The repertoires of communicatory and some other behaviours are incomplete and the social significance of play in lorises is totally overlooked. Moreover, certain interpretations are suspect or erroneous—for example, that concerning the defensive posture of *Arctocebus*, and the view that the primary function of the prosimian dental comb is gum collection rather than grooming, despite the indisputable importance of the former function in *Euticus*, so clearly shown here.

The title, if not the subtitle of the book could be misleading if, by it, one were led to expect a thorough-going review of our knowledge of all nocturnal primates. In this respect, the work is erratic and there are some surprising omissions, even within the narrower compass of the Lorisidae. A concluding chapter does attempt to place nocturnal primates among the diurnal forms in a wider perspective, but here again the

quality of the result is decidedly uneven, marred by the dubious validity of a number of contentions regarding Primate evolution and ecology. Thus, the idea that competition for food is necessarily eliminated or even reduced by a Box-and-Cox temporal partitioning of an ecological niche does not stand up to close scrutiny as a general truth.

The strength of this book, and it is indeed strength, lies in the author's approach to, and resolution of, the problems of behavioural ecology in his Gabonese prosimians; and in his demonstration that no aspect or detail of structure or behaviour is arbitrary, without meaning in terms of the animal's survival. That is reason enough to commend this volume.

Gilbert H. Manley

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Sound waves in solids

Sound Waves in Solids. By H. F. Pollard. Pp. 366. (Pion: London; Academic: London, 1977.) £10.50.

THE author of this latest addition to the rapidly growing literature of acoustics is Dr Howard F. Pollard who is an Associate Professor in charge of the Physical Acoustics Laboratory at the University of New South Wales in Sydney, and is the leading Australian worker in this field. The objective of the author, as indicated in the introduction, is to provide initially the necessary theoretical background for the reader to appreciate fully the various applied fields of acoustics, such as non-destructive testing. This practical aspect is kept well to the fore throughout the book, and uniquely the author introduces, after the basic theory, synopses of experiments which appropriately illustrate the problem under discussion. A particular usefulness of the text to the student is the provision of sets of questions at the end of each chapter which are based on the problems included at the end of specified sections of the foregoing subject matter. A full set of solutions to the book.

In considering the general contents of the book, it is the chapter on acoustic waveguides on which the author can be particularly congratulated. He has introduced this important topic through the simpler system of a fluid waveguide, and, having established the essential theory and con-

cepts, he deals with the understanding of the various practical applications. The chapter on ultrasonic experimental techniques is quite comprehensive and the latest developments such as ultrasonic spectrometry receive adequate recognition. The author's own research involvement in such work enriches his presentation of the subject; this also applies to the chapter in the book concerned with the exciting field of acoustic visualisation. A representative number of holographic applications are given, and here the author has revealed his musical interests by the inclusion of some reconstructions at audiofrequencies.

The problem of the choice of references is difficult in a field like solid-state ultrasonics, which is large and rapidly expanding, but the author has helped the reader by including the appropriate references at the end of each chapter. Moreover, those concerned with particular experiments are given with the text. An author and a subject index are also included. The line diagrams are very clear and also the text, although the use of smaller print for experimental descriptions gives an impression of over compression.

The title is indicative of the breadth of the subject so efficiently and clearly discussed by the author, and the book deserves to be welcomed alike by students, teachers and research workers.

R. W. B. Stephens

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newly on the market

These descriptions are prepared by the staff of *Nature* on the basis of material provided by manufacturers. The Reader Enquiry Card faces the inside cover.

Microprocessor-controlled logger. Pye Unicam. The Philips PM 4000 system is designed for logging voltages, currents and temperatures; later models will handle mechanical (strain) measurements directly. Up to 950 channels can be controlled by one unit, and remote control and operation of the data logger are possible. The main-frame unit can hold up to 50 channels with extension units for each additional 100 channels. Extension units can be up to 10 m from the control unit—the use of digital communication between mainframe and extension units eliminates noise problems. Results can be conditioned into the relevant engineering units using internal linearisation tables and formatted for internal or external presentation. Programming is reduced to a simple question-and-answer dialogue using a built-in keyboard and integral alphanumeric display, and no special programming language is necessary. Completed programs can be dumped out to, or dumped in from, a digital cassette recorder enabling custom program libraries to be built up.

Circle No. 45 on Reader Enquiry Card

Single-test creatinine analyser. Beckman. The new Creatinine Analyzer-2 automates precise measurements of creatinine in plasma, serum, urine, or amniotic fluid. Time from sample injection to final readout is 26 s compared with the 15 min usually required for running the test manually. Creatinine is clinically important in determining kidney function, in monitoring renal dialysis therapy, and as a warning of incipient muscular disease such as muscular dystrophy and hypothyroidism. Operation of the Creatinine Analyzer-2 is based on the Jaffe reaction. Colour is generated as the patient sample mixes with reagent under controlled temperature conditions, and the analyser measures the rate of colour change, which is a more precise indicator of creatinine than endpoint analysis.

Circle No. 46 on Reader Enquiry Card

Sample-handling system for radioimmunoassay. Beckman. The modular Phase I RIA sample-handling system is designed to improve radioimmunoassays by simplifying procedures for rapid, accurate, inexpensive analyses. Stand-alone instruments, accessories, and packaged RIA kits make up the system, which is available as a whole or in parts. Phase I system includes the Gamma 4000 gamma spectrometer, which is capable of processing up to 400 samples automatically; an on-line DP-5000 microprocessor data reduction system, which stores up to 10 RIA data reduction programs including counts per minute; the Model J-6 Centrifuge; and an all-in-one pipettor/diluter/dispenser which allows the solid-phase Beckman RIA Kits to be run with only one simple pipetting step. Basic to the Phase I System are Beckman double-antibody, solid-phase RIA kits, which reduce incubation time, centrifugation requirements, and chance for error.

Circle No. 47 on Reader Enquiry Card



Beckman RIA system

Blood bank refrigerator. Forma. The Forma Scientific model 3880 +4 °C blood bank refrigerator has a 300-bag capacity and features top-to-bottom temperature uniformity within 0.5 °C in compliance with nationally recognised standards. Standard equipment includes six 50-bag capacity roll-out storage drawers with label holders, a self-charging battery alarm system with alarm ringback and remote alarm contacts, a pressure writing temperature recorder, automatic condensate removal (to eliminate drain lines), and a 100% stainless steel interior cabinet with cabinet lights. The unit is 30 inches wide, 28 inches deep, and 73 inches high.

Circle No. 48 on Reader Enquiry Card

Thermomechanical analyser. Perkin-Elmer. The Model TMS-2 thermomechanical analyser when used for the thermal characterisation and quality control of materials, determines precisely and rapidly, dimensional changes (expansion and extension modes) or visco-elastic changes (penetration and compression modes) of small samples in any form—powder, pellet, film, fibre or moulded part. The Model TMS-2 system consists of analyser, analyser control and heater control modules. Displacement can be recorded simultaneously as either integral or derivative signal, both individually attenuated and filtered. The linear variable differential transformer is in complete thermal isolation from the furnace, which permits operation at very high sensitivity with flat baselines over a broad temperature range. Heating and cooling rates from -170 °C to +325 °C are possible with the standard furnace and an optional low temperature furnace is available. A sliding furnace assembly, positive positioning of probe on sample and open tube design facilitates ease of sample handling.

Circle No. 49 on Reader Enquiry Card

Automated blood gas analyser. Technicon. The Technicon BG II, pH/blood gas system uses an advanced, built-in computer to help provide accurate answers on eleven parameters on blood samples as small as 130 µl. Small samples give the instrument wide application in hospital critical care facilities where patients with severe burns and other major illnesses are treated. These patients, as well as infants and the very elderly, have frequently been incapable of giving a large enough sample in the past. The Technicon BG II system is extremely easy to operate and maintain. With the BG II, the hospital can have cost-effective coverage in the central laboratory as well as in any other areas where blood gas samples are processed. The results of the eleven tests are available in 2½ min, and are printed out on paper tape or multiple-copy ticket. The values for pH, PCO₂, PO₂, and haemoglobin are measured directly; standard base excess, base excess, standard bicarbonate, bicarbonate, O₂ saturation, O₂ content, and total CO₂ are computed and printed out simultaneously.

Circle No. 50 on Reader Enquiry Card

Electronic precision balances. Mettler. A compact, rugged housing is used for two new electronic precision balances. The PL1200 can weigh up to 1,200 g with a readability of 0.01 g and the PL200 can weigh up to 220 g with 0.001 g readability. An automatic stability detector circuit monitors the zero point and indicates when a reliable weighing result is available for reading. The measuring cycle can be adjusted from the outside in four different steps. This allows the balance to adapt to difficult weighing problems (such as restless lab animals) and to prevailing environmental conditions without affecting its weighing performance. The capabilities of weighing below the balance and of measuring moisture loss with the help of the LP12 infrared dryer are also built into these balances. Printers, weight-count converters and other peripheral instruments can also be connected by means of a BCD output.

Circle No. 51 on Reader Enquiry Card



Mettler PL1200

Interferometer. Metals Research. The new Metals Research interferometer has been developed for the examination of the transmission characteristics of laser rod materials both for zonal selection and final quality assessment. Following the classical lines of the Twyman Green Interferometer the instrument is packaged so as to provide a sample space of 40 mm diameter, 200 mm long. Folding the light path in an optimum manner gives a table-top instrument of only 20×11×10 inches, weighing approximately 110 pounds. Accuracy is 1/20 wave. Special design features include built-in laser illumination to provide high contrast fringes for critical viewing of small apertures (6 mm dia.), and a decohering filter (rotating disk) to suppress spurious fringes created within the system. A built-in Polaroid roll film holder takes 1:1 photographs of specimen patterns.

Circle No. 52 on Reader Enquiry Card

Automatic analyser for nitrogen oxides. Pye Unicam. The Philips PW 9762 analyser continuously and automatically measures atmospheric oxides of nitrogen (NO, NO₂, or their sum NO_x) in a simple and economic manner, and it will do so for up to 3 months without intervention. NO and NO_x are both measured in identical samples of ambient air, NO₂ initially being qualitatively reduced to NO for the measurement of NO_x (NO₂ is the difference between measured values of NO_x and NO). This use of identical sampling eliminates false negative values of the NO_x signal. The PW 9762 makes use of the chemiluminescence principle which creates a reaction between NO and O₃ and, by providing an in-built ozone generator, the Philips analyser obviates the need for an external supply of gas—such as, for example, oxygen. Highly-sensitive pulse-counting techniques enable the analyser to be used in normal atmospheric-pressure conditions, thereby overcoming one of the earlier drawbacks of the chemiluminescence principle which demanded near-vacuum working conditions. The PW 9762 is extremely compact and fits into a standard 19-inch racking system. It incorporates its own sample pump and power-supply unit and can be ready for use within 20 min of connecting up.

Circle No. 53 on Reader Enquiry Card

Anaerobic work centre. Forma. Forma Scientific introduce a completely self-contained anaerobic work centre designed to add immediate anaerobic capabilities to clinical and research labs of all sizes. The work centre consists of a large-capacity glove cabinet with a highly visible work area; a built-in incubator with solid-state temperature control and a forced-draft air flow system to maintain a comfortable working environment; and a front access auto-sequence interchange with single, push-button aerobic to anaerobic conditioning for quick-in, quick-out transfer of anaerobic cultures and apparatus. The work centre is complete with regenerative palladium catalyst and dessicant dryer modules, de-ox indicators, disposable work pads, and a waste removal system. Accessories available include an anaerobic collection/transfer cart, add-on work modules, inventory racks, and a mobile instrumentation bench for set-up of an integrated anaerobic station, including a low noise, oilless vacuum pump, built-in CO₂ and N₂ gas bottle platform, undercounter storage cabinets, and dual-wheel casters for easy moving.

Circle No. 54 on Reader Enquiry Card

On-line chemical analyser. Kevex. The Kevex material analyser is intended for rapid and non-destructive on-line measurement of chemical compounds. The analyser includes a pre-programmed microprocessor capable of calculating the concentrations of up to 19 different elements simultaneously in as little as 5–10 s. Applications include routine quality control of fluids and slurries by batch sampling tests or continuous on-line flow analyses with the option of closed-loop control. Provisions for data logging and external signals for initiating element concentration correction or product rejection are included. The system can be fully automated.

Circle No. 55 on Reader Enquiry Card



Kevex material analyser

Low-temperature temperature controllers. Neslab. Exatrol and Cryotrol controllers are designed primarily to extend the usefulness of the Neslab immersion coolers by providing the ability to maintain constant temperatures at any point up from the lowest point attainable. Neslab CryoCool and Bath Cooler immersion coolers can now be operated to maintain constant temperatures from as low as -100 °C. The Exatrol temperature controller offers temperature stability of better than ±0.05 °C when used with a Neslab CryoCool or Bath Cooler. Linear cooling or heating rates can be programmed by adding the ETP-3 electronic temperature programmer. The Cryotrol model provides temperature stability of ±0.5 °C. This economical method can be used where less critical temperature control is needed. A constant low temperature bath, as well as programmed freezing rates, is now possible with this new series of temperature controllers.

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